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**PROVISIONAL SPECIFICATION FOR AN INVENTION
ENTITLED**

Invention Title: FOR16D PROTEINS, NUCLEIC ACIDS AND
METHODS BASED THEREON

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The invention is described in the following statement :

FIELD OF THE INVENTION

This invention relates to the field of cancers and in particular to nucleotide sequences of the fragile site FRA16D, of the FOR16D gene and amino acid sequences of its encoded proteins, as well as derivatives and analogs thereof and agents capable of binding
 5 thereto, and uses of these, such as in diagnosis and therapy.

BACKGROUND OF THE INVENTION

Cancers are a significant factor in mortality and morbidity, with onset rates of forms of cancer being quite high in all places of the world. Early detection greatly improves the
 10 chances of remission and considerably reduces the chance of the cancer metastasizing. The treatment of early stage cancers is also much more benign so that there are less severe residual effects resulting from the treatment. Accordingly early detection of cancers is a high priority in management of the diseases. Similarly treatment of various cancers are of mixed outcome and it is desirable to provide for alternative treatments at
 15 least for certain forms of cancers.

Cancers are of many different types and severity, however the uncontrolled proliferation of cancers cells is invariably associated with damaged DNA of one form or another. Some types of cancer are familial in the sense that there is an increased risk of
 20 contracting cancer, but the hereditary characteristics in most cancers are not simple and there is only usually a few fold increased risk among family members as compared to the general population. The DNA damage in most cancers are associated with somatic mutations the acquisition of which is thought to be associated with exposure to certain environmental factors.

25 A very large number of genes have been identified as being associated with the onset of cancer and this reflects the complexity of the regulation of normal cellular proliferation. These genes can be categorised into three groups a first of which includes the so called oncogenes or protooncogenes which are often associated with positive control
 30 elements, enhancing cellular proliferation in the normal cellular cycle. Certain mutations in these positive control elements trigger uncontrolled proliferation. A second group are the so called tumour suppressor genes, which are genes that normally suppress proliferation, and inactivation or reduction in activity of these leads to abnormal proliferation. These tend to act in a recessive fashion. A third group are the
 35 so-called mutator genes which are normally responsible for maintaining genome integrity during the proliferative cycle, and if these are defective then the general mutation rate increases and the consequent chance of providing for a transforming mutation increases.

One mapping technique to locate the site of chromosomal lesion in a cancer cell is known as the loss of heterozygosity (LOH) technique. Eukaryotes have two copies of each chromosome, apart from the sex chromosomes, and as a result cancers that result from mutations in a tumour suppressor generally require two mutations. Sometimes one
 5 mutation will be inherited, and a second mutation is required to trigger the cancer leading to loss of function of both copies of the gene in the individual. Quite often these secondary mutations will be deletions and their location can be detected by checking the presence of highly polymorphic genetic markers from the tumour tissue and from another site such as blood. The markers that are heterozygous in normal
 10 tissue and have become homozygous in the cancer tissue can give an indication of the lesion concerned.

The LOH technique is however quite difficult to routinely perform and interpret reliably, this is particularly so because any tumour sample usually is also contaminated
 15 by non-tumour tissue, and it is at times difficult to distinguish because of a decreased relative intensity, and quantitative amplification techniques will often need to be employed. Another limitation relates to the availability of a suitably dense array of markers which generally leads to the detection only of larger deletions. A single tumour may have LOH in many distinct regions, but LOH will only be detected in those
 20 regions that have been tested.

The use of these LOH studies have identified a number of sites some of which correspond to regions of the chromosome termed fragile sites.

25 Fragile sites have been proposed to have a determining role in cancer associated chromosomal instability. There are in excess of 100 fragile sites in the human genome of which the fragile site *FRA11B* is located within the *CBL2* proto-oncogene (Jones *et al.*, 1994, 1995) and the *FRA3B*, *FRA7G* and *FRA16D* sites have been located within or adjacent to regions of instability in cancer cells (Ohta *et al.*, 1996; Sozzi *et al.*, 1996;
 30 Engelman *et al.*, 1998; Huang *et al.*, 1998a,b).

There are two distinct forms of chromosomal anomaly referred to as fragile sites (Sutherland *et al.*, 1998)). The 'rare' form is polymorphic in the population and is accounted for by the expansion of repeat DNA sequences beyond a copy number limit.
 35 The 'common' form is present at many loci in all individuals. Despite determination of the complete sequence analysis of the common fragile site, *FRA3B* (Boldog *et al.*, 1996; Inoue *et al.*, 1997; Mimori *et al.*, 1999) and the partial sequence analysis of the common fragile sites, *FRA7G* and *FRA7H* (Huang *et al.*, 1998a,b; Mishmar *et al.*, 1998) the molecular basis for common fragile sites is not yet understood.

Fragile sites are also distinguished by the culture conditions required for their induction. Common fragile sites are (mainly) induced by aphidicolin, whereas the rare fragile sites are induced by either high or low concentrations of folate or the AT-rich binding chemicals such as distamycin A or by bromodeoxyuridine. The role of chromosomal fragile sites in human genetic disease was thought to be restricted to fragile X syndrome caused by the *FRAXA* fragile site, however a mild form of mental retardation has been associated with *FRAXE* and the *FRA11B* fragile site appears to predispose to 11q breakage leading to some cases of Jacobsen syndrome.

Recent detailed molecular analysis of fragile site loci has demonstrated that the common fragile site *FRA3B* is located within a region subject to localised deletion and that this deletion is frequently observed in certain forms of cancer (Ohta *et al.*, 1996; Sozza *et al.*, 1996). *FRA3B* lies proximal to the major region of LOH on chromosome 3p previously shown to be responsible for deletion of the *VHL* tumour suppressor (Gnarra *et al.*, 1994). The cancer-associated *FRA3B* deletions can result in inactivation of a gene (*FHIT* -Fragile Histidine Triad) which spans the fragile site (Croce *et al.* US patent 5928884). The *FHIT* gene product has been shown to have a role in tumour growth (Siprashvilli *et al.*, 1997) but quite what the significance or nature of that role is subject of active research at the present.

Another common fragile site *FRA 7G* has also been shown to be located within an about 1Mb region of frequent deletion in breast and prostate cancer (18,19) as well as squamous cell carcinomas of the head and neck, renal cell carcinomas, ovarian adenocarcinomas and colon carcinomas (20). The human caveolin-1 and -2 genes are located within the same commonly deleted region as *FRA 7G*. Caveolin-1 has been shown to have a role in the anchorage dependent inhibition of growth in NIH 3T3 cells (21). The caveolins are therefore candidates for the tumour suppressor gene presumed to be located in the *FRA 7G* region (20).

Another common fragile site which is aphidicolin inducible is the *FRA16D* site. *FRA16D* has been localised at 16q23.2. within a large overlapping region of chromosomal instability in breast and prostate cancer as defined by loss-of-heterozygosity (24,25). One study has found that a significant proportion (77%) of breast cancers carries a deletion at 16q23.2, including the marker D16S518 in the immediate vicinity of *FRA16D* (24).

There has been no characterisation of a nucleic acid or protein associated with the *FRA16D* site and the physical location of *FRA16D* has not yet been determined. Such

a characterisation is desirable to enable potentially early diagnosis and assessment of risk as well as potentially providing for a therapeutic treatment.

SUMMARY OF THE INVENTION

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The inventors have produced a detailed physical map of the *FRA16D* region which provides markers to identify a relationship between this fragile site and DNA instability in neoplasia and which, further, may allow better diagnosis of cancers associated with the region. This analysis reveals the existence of an intimate relationship between the location of *FRA16D* and homozygous deletions in various tumours, culminating in the coincidence of two tumour cell DNA breakpoints with the most likely position of the fragile site.

15 The inventors have also characterised the nucleic acid associated with *FRA16D* especially by nucleic acid sequencing. Analysis of the DNA sequence has identified a number of introns and exons which are found to exist in four different splice variants of what will be termed protein FOR16D. RNA analysis has also been conducted and thus far two species of mRNA associated with the region have been detected.

20 In a first aspect the invention could be said to reside in a method of detecting genetic variations of a 16q23.2 target in the 16q23.2 region of the chromosome, said method comprising the steps of contacting target nucleic acid with one or more oligonucleotides suitable for use as hybridisation probe or PCR priming specific for binding the 16q23.2 specific target, and ascertaining the binding of said oligonucleotide.

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It will be understood from the specification that the 16q23.2 specific target might be selected to be within the group comprising the FOR16D gene, the *FRA16D* site, or mRNA encoding FOR16D protein or all of these collectively. The target may include chromosomal rearrangements and mutations thereof and the rearrangements or mutations may, in one form, be cancer associated. The variations may include markers in the region such as set forth in this specification including in figures 1, 2, 7 and 8.

35 The 16q23.2 target within the FOR16D gene might be selected from one or more of the group comprising exons a, 1, z, w, 2, 3, 4, 5, 6 or x or introns located therebetween or control elements in other adjacent regions that effect an altered expression of the FOR16D gene. Such adjacent regions may have a promoter, enhance elements or other regulatory elements. The target may be any one of the splice variants currently identified as FOR16DI, FOR16DII, FOR16DIII or FOR16DIV or it might include other combinations of two or more of the exons.

It is noted in particular that breakpoints of three out of five 16q23.2 translocations associated with multiple myeloma map within the alternate splice of this FOR16D intron, that is, between exons 4 and x, and in one form a preferred target is the intron between exons 4 and x or a portion thereof.

In some circumstances the method might be used to detect any rearrangements in a larger target area. Thus it might be desired to use a plurality of oligonucleotide which might be selected to bind to a range of target binding sites within the 16q23.2 specific target to detect for a range of changes. This might be used for example to detect for chromosomal rearrangements such as deletions within the FRA16D site or beyond that in the broader 16q23.2 region. The plurality of oligonucleotides or a plurality of specific binding sites of the 16q23.2 target are preferably spacially separated so that binding of each of the plurality of oligonucleotides or binding to the plurality of specific binding sites can be separately ascertained. The spacial separation might, for example, be conveniently provided as an array on a solid support, for example in a form that is common referred to as a gene chip (see for example patent specifications US 5288514 and US 5593839). Instead of a plurality of oligonucleotides it may be desired that the target be probed by a single oligonucleotide.

Alternatively the target area might be small, thus for example the method might be used to ascertain the presence or absence of a particular mutation or allelic variation in the 16q23.2 target. Thus for example a target of the z, w, 5 or 6 or x exon will distinguish between FOR16DI, FOR16DIV, FOR16DII and FOR16DIII transcription variants. A small target area might also be adequate for use with gross chromosomal rearrangements in so far as this might be used to determine the presence or absence of junctions of known chromosomal rearrangements, or alternatively the binding or non binding of one or more of a plurality of oligonucleotides. The target area might also be selected to allow for assessment of the presence or absence of cancer associated point mutations or small DNA rearrangements, using suitably selected oligonucleotides.

The base sequence of the oligonucleotide chosen will depend upon several factors known in the art. Primarily the sequence of the oligonucleotide will be determined by its capacity to bind to the target nucleic acid sequence. The nature of the sequence will depend to some extent on the stringency of the hybridisation required, and whether or not it is desired for one oligonucleotide to detect variation in sequence or not. If variation in one nucleotide is required the stringency of the hybridisation will be high. The length of the oligonucleotide will also be determined by the stringency of the reaction required.

- The binding might be by *in situ* hybridisation of a chromosomal spread, or other suitable spacial arrangement of the target region such as for example on a so called gene chip. Such hybridisation methods will generally provide for an oligonucleotide and be
- 5 capable of binding the target over a span of at least 15 nucleotides. In the case of hybridisation techniques the oligonucleotides will generally carry a label which can be detected by known measuring methods, especially when bound to the 16q23.2 target. Such labels might include radiolabels such as ^{32}P or a fluorescent marker.
- 10 The method might require a preamplification step whereby the target nucleic acid is amplified, to make it easier to ascertain the binding or non binding of the nucleic acid to the target site.
- On the other hand the oligonucleotide might be suitable for amplification of a segment
- 15 of the target nucleic acid such as by PCR, in which case the size of the target may be somewhat different. With this variation two oligonucleotides might be selected, to provide for amplification of at least part of the target nucleic acid, at least one of the oligonucleotides is required to bind in the target.
- 20 The target nucleic acid might be presented in any one of a number of physical forms. Nucleic acid from an individual might be isolated and perhaps digested by a restriction enzyme and spread out such as by electrophoresis on an agarose or polyacrylamide gel, so that binding of the oligonucleotide can be effected whilst the target nucleic acid is supported by the gel or this might be supported on other solid medium such as a gene
- 25 chip or a metaphase chromosomal spread. Alternatively the oligonucleotide or oligonucleotides might be fixed, and the target nucleic acid might either be diminished in size, or not, and then binding of fragmented targets to the fixed oligonucleotide determined.
- 30 The target nucleic acid might be in the form of chromosomal DNA, or might be cDNA or mRNA.

- This method might also be used to detect other variants, homologs or analogs of the FRA16D site, FOR16D gene, or other nucleic acid sequences disclosed in this
- 35 specification. Thus it might be, for example desirable to determine analagous gene in livestock, domestic, laboratory or sporting animals. Alternatively one might wish to determine another analogous protein that plays a similar role in humans.

In a second aspect the invention relates to a method of detecting the number of alleles for one or more markers in the 16q23.2 target, and this may be a means of perhaps providing a measure of the loss of heterozygosity in an individual. This aspect of the invention therefore relates to locating a deletion that overlaps with the FRA16D region.

- 5 The method might be achieved by providing a first set of one or more oligonucleotides and a second set of one or more oligonucleotides the first set of oligonucleotide being specific for a first variant of the target nucleic acid, the second set of oligonucleotides being specific for a second variant of the target nucleic acid, the first and second set of oligonucleotides being labelled so as to be capable of being distinguished, and the
- 10 method comprising the steps of comparing the proportion of binding of the first and second set of oligonucleotides. A method of this sort is set forth in US patent specification 5928870 to Lapidus *et al*, which for purposes of practicing the invention is incorporated herein by reference.
- 15 It will be understood that the above method is useful in categorising the risk of contracting certain types of cancer associated with the FRA16D fragile site or other portion of the 16q23.2 region.

- In a third aspect the invention could be said to reside in a method of determining the
- 20 level of expression of the FOR16D gene or any one or more exon thereof, by determining the level of mRNA expression using a probe specific for the FOR16D gene or exon thereof. This might be used to determine the dysregulation of FOR16D expression. It will be understood that it may be desired to also determine the level of expression of variants of the gene or exons including rearrangements and mutants
- 25 including those associated with cancers. This is likely to give a prognosis in relation to at least certain cancers that are currently contracted or perhaps an indication of the risk of contracting one or more types of cancer.

- In a fourth aspect the invention could be said to reside in an isolated nucleic acid
- 30 molecule selected from the group comprising
- a) nucleic acids sequences disclosed in the figures hereto or parts thereof
 - b) FRA16D site
 - c) FOR16D gene, or exons thereof
 - d) mRNA of the FOR16D gene
 - 35 e) cDNA of the FOR16D gene
 - f) variants of the above including, chromosomal rearrangements and mutations of sequences set out in a) to e) including those variants associated with cancers

- g) nucleic acid sequence capable of hybridising specifically to any sequence of a to e above or its complement, and especially those capable of doing so under stringent conditions.

5 The nucleic acid molecule might include a mosaic from within the above molecules such as a combination of two or more of the group comprising the following, exon a, 1, z, w, 2, 3, 4, 5, 6, or z or introns located therebetween or control elements in other adjacent regions that effect an altered expression of FOR16D, and it will be understood that such a mosaic includes a molecule encoding cDNA of variants of the FOR16D
10 protein, whether a wild type allele, a mutated version, or otherwise rearranged. It will thus be understood that the invention includes antisense molecules to any regions of control that might be contemplated above. Such antisense molecules may be used to vary the expression of such protein as are produced by the FOR16D gene or perhaps adjacent genes such as the c-MAF gene.

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It will be understood that such nucleic acids include portions of nucleic acids that are suitable for use as primers or probes.

20 The invention may also be said to include nucleic acids encoding a tumour associated gene from a human or animal capable of hybridizing with any nucleic acid of the fourth aspect of the invention.

25 In a fifth aspect the invention could be said to reside in a recombinant vector including one or more nucleic acid sequences as set out above, and preferably operably linked to a control element such as might include a functional promoter. The recombinant vector might be used as an expression vector to produce or overproduce FOR16D protein or variants thereof, or perhaps overproduce nucleic acids associated with the FOR16D gene such as an antisense molecule. Suitable vectors are generally available commercially or may be constructed as described elsewhere or as is known in the art.

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In a sixth aspect the invention could be said to reside in an isolated protein molecule, the protein molecule being selected from the group comprising the following:

- a) a FOR16D protein, or
- b) a mutant or variant FOR16D protein which might optionally be
35 associated with a cancer

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In a seventh aspect the invention could be said to reside in a polypeptide produced by any two or more exons selected from the group comprising a, 1, z, w, 2, 3, 4, 5, 6, x joined, said exons being either as complete exons or partial, and may be variants.

The invention might also encompass a purified cancer associated protein including a string of amino acids unique to a FOR16D protein and more particularly as set out in any one of figures 13 A to D, preferably said amino acid string being at least 10 amino acids long and exhibiting at least 70% amino acid homology more preferably at least 90% homology.

The protein may have an oxidoreductase domain or may have a role in DNA replication of chromosomal division.

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In one form the purified cancer associated protein includes an amino acid string with an amino acid sequence homology of greater than 70% but more preferably greater than 90% with the amino acid string LPPGWEERT, and is associated with DNA replication or chromosomal division. Such a purified protein may be used for treatment of certain cancers.

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In another form the purified cancer associated protein includes an amino acid string with an amino acid sequence homology of greater than 70% but more preferably greater than 90% with an amino acid string selected from the group comprising:

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VVVVTGANSIG, MTLDLALLRSVQ, PLDVLCNAA and
VNHLGHFYL.

In an eighth aspect the invention includes an agent capable of selectively binding a FOR16D protein or fragment or variant thereof. Such agents may be particularly useful in diagnostic methods. Such an agent may also be used to bind a protein containing a string of amino acids unique to FOR16D or variant thereof and in particular such variants that are currently known to be associated with one or more forms of cancer. The agent may selectively bind to the variant FOR16D as compared to an FOR16D protein not associated with cancer. Such an agent might be an agonist or an antagonist of FOR16D function. It might therefore be desired to provide for a number of agents each capable of selectively binding to a separate one of a number of variants of FOR16D so that it is possible to distinguish between variants. Thus for example it might be desired to target the C terminus of respectively FOR16DI, FOR16DII, FOR16DIII and FOR16DIV to distinguish between these three proposed forms. The invention therefore also encompasses a method of detecting variants of the FOR16D protein. Measuring the relative levels of these four and other forms of FOR16D protein is likely to give an indication of regulatory perturbations which may be associated with certain cancers.

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The nature of the agents can vary depending on their intended use. Thus for a diagnostic method an antibody or fragment thereof, such as an Fab fragment, of a recombinant molecule carrying the variable region of an antibody recognising the desired portion of the FOR16D may be adequate. The antibody might be polyclonal
 5 however preferably the antibody is a monoclonal antibody prepared by known techniques.

Alternatively small molecules capable of binding the desired portion of the FOR16D protein may be used, such small molecules might include peptides, proteins, nucleic
 10 acids or sugars or other organic molecules. These can be isolated by screening using known techniques from libraries of suitable compounds. Such small molecules can then be tested for antagonist or agonist properties to potentially provide a therapeutic agent which have the potential to be used in the treatment of cancers. These agents would be administered by clinicians in an appropriate manner.

15 Also useful therapeutically might be the provision of an isolated protein of the seventh aspect of the invention, particularly those forms that mimic the action of a wild type FOR16D, and perhaps simply the purified FOR16D. It is anticipated that the FOR16D protein in at least one of its forms is a tumour suppressor, that is, its absence increases
 20 the risk of aberrant cell division leading to a cancer. Accordingly one form of therapy may include the administration of such a protein to an individual who is considered at risk, particularly if they are found to have a faulty FOR16D protein. Such administration would be in conformity with normal practices in a suitable excipient. It may also be the case that the aberrant FOR16D protein actively enhances
 25 tumourigenesis and accordingly it might be appropriate to administer an antagonist of the aberrant variant at the same time. Alternatively the administration of the antagonist on its own may be of therapeutic benefit.

Another form of treatment which is becoming increasingly contemplated is to provide
 30 for a method of gene therapy and one method of undertaking cell therapy is to provide for certain progenitor cells which include incorporated therein a vector capable of producing an appropriate form of FOR16D protein. Accordingly a ninth aspect the invention could be said to reside in a recombinant host cell having stably inserted therein DNA of any one of the forms of DNA contemplated in the third aspect of the
 35 invention. In preference the DNA is capable of producing a tumour suppressing form of FOR16D, and most conveniently this will be a wild-type form of FOR16D, which may simply be a cDNA molecule or the FOR16D gene. Alternatively however it may also be desired to have a host cell which has a DNA sequence capable of producing an antisense molecule in the case where an aberrant tumour promoting form of the

FOR16D molecule is produced by the individual to be treated, the antisense capable of reducing the level of expression of the FOR16D molecule.

Methods of gene therapy are not limited to cases where the appropriate nucleic acid is delivered in a host cell, but also includes the administration of the nucleic acid specifically to the site of interest.

The recombinant host cell may not necessarily be used for therapeutic purposes, it may also be used for over-expression of the protein, or a nucleic acid associated with FOR16D, or the 16q23.2 region, and may therefore be bacterial, yeast, plant, animal, preferably mammalian or human.

Additionally the invention contemplates the provision of a transgenic non-human animal carrying recombinantly altered or overexpressing 16q23.2 DNA, preferably FRA16D or FOR16D gene, or other DNA of the fourth form of this invention. The recombinant DNA might be incorporated into the chromosome of the host, alternatively the host cell may carry said recombinant DNA in a self replicating element such as a plasmid.

The agents of the eighth embodiment may be used for level of expression of FOR16D, variants or exons thereof, to determine whether there is an altered level of expression. Thus a western blot using a labelled agent may be used for the purpose using known techniques. This is another means of measuring dysregulation of expression.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1: Positional cloning of *FRA16D* and location of loss of heterozygosity and translocation in cancer.

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A. The locations of loss-of-heterozygosity regions in breast and prostate cancer and the approximate location of the *FRA16D* fragile site are indicated with respect to genetic markers (downward arrows) in the 16q23.2 region. Markers in the vicinity of *FRA16D* are shaded. The approximate location as determined by Chesi *et al.* (1) of multiple myeloma breakpoints and the *c-MAF* gene (bar) are also shown by upward black arrows. Not to scale.

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B. Map of the contig of YAC subclones across the *FRA16D* region with respect to genetic markers and *FRA16D*. Open boxes indicate those YACs which map by fluorescence *in situ* hybridisation proximal to

FRA16D, grey boxes are those which span *FRA16D* and black boxes indicate those YACs which map distal to *FRA16D*. Not to scale.

Figure 2: Positional cloning of *FRA16D* and the extent of heterozygous and homozygous deletion in the AGS tumour cell line.

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A. Pulsed-Field gel map of ~1Mb of the 'Right Hand Side' (RHS) of YAC My801B6 and the location of BACs, genetic and STS markers (key markers are boxed). Restriction sites between Afma336yg9 and WI2755 are shown in B. The AGS stomach cancer cell line homozygous deletion is indicated - shaded circles denote the presence and open circles the absence of PCR products for the STS markers. Maximal region of heterozygous deletion in AGS cell line is indicated by polymorphic D16S518 and D16S3029 PCR products, indicated as A and B alleles. The two AGS cell line chromosome 16s are indicated by shaded bars.

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B. Restriction map of the critical *FRA16D* region (Afma336yg9 to D16S3029) showing the location of key members of the lambda subclone tile path used for FISH in figure 3. Clones designated λ -n are from 325M3; others are from 801B6. Open boxes represent those subclones found to map proximal (on the basis that >85% of their FISH signals were proximal to *FRA16D*), grey boxes those which appear to span the fragile site (less than 85% on one side or other of *FRA16D*) and black boxes those which are distal to the fragile site (on the basis that >85% of their FISH signals were distal to *FRA16D*). λ clones which gave high background on FISH were not scored. These and other λ clones for which FISH data were not obtained are included as thin boxes. STS localisation of the AGS homozygous breakpoints are indicated by the presence (shaded circles) and absence (open circles) of PCR products.

Figure 3: Fluorescence *in situ* hybridisation (FISH) of lambda subclones against *FRA16D* expressing chromosomes.

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Each panel contains two *FRA16D* expressing partial metaphases, with and without FISH signal merged. In each case the width of the gap or break at the fragile site is greater than the width of the chromatid. (a) λ 504 showing signal proximal to *FRA16D*; (b) λ 181 showing signal

proximal and distal to *FRA16D*; (c) λ 191 (upper) and λ 8 (lower) showing signal distal to *FRA16D*. Images of metaphase preparations were captured by a cooled CCD camera using the ChromoScan image collection and enhancement system (Applied Imaging Int. Ltd.). FISH signals and the DAPI banding pattern were merged for figure preparation.

Figure 4: Fluorescence *in situ* hybridisation mapping of the lambda subclone tile path across *FRA16D*.

The individual lambda clones were scored against chromosomes where the *FRA16D* gap or break was greater than the chromatid width. Each increment represents a single FISH signal. n = number of chromosomes scored. Scores were plotted as proximal (p) and distal (d) with respect to *FRA16D*. Maximum location for *FRA16Ds* indicated by arrows. Location of BAC clones 325M3 and 353B15 is also shown. The boxed lambda contig subclones indicate those for which FISH signal results with respect to the *FRA16D* fragile site were obtained - open boxes, had >85% signal proximal to *FRA16D*; grey boxes, spanning (<85% signal on one side or other of *FRA16D*) and black boxes, had >85% signal distal to *FRA16D*. While this figure is not to scale the location of the lambda clones can be determined from their position in figure 2. Thin boxed lambda clones are those for which FISH data was not obtained.

Figure 5: Duplex PCR deletion detection at the *FRA16D* locus in tumour cell lines.

PCR products from the duplex of STSG-10102 and dystrophin DMD Pm were subjected to agarose gel electrophoresis and ethidium bromide staining. Template DNAs were seven tumour cell lines and blood bank and no DNA controls. Markers are *Hpa*II digested pUC19. The position of the STSG-10102 and DMD Pm PCR products are indicated by large grey-filled arrows while the primer dimer PCR artefact is indicated by a small white arrow.

Figure 6: Is a diagrammatic representation of *FRA16D* transcripts with respect to *FRA16D* and common homozygous deletions. A. summarises the data in figure 2. B shows the position of two BACs and below that shows the DNA sequences that have been obtained. C. shows three of the four

predicted variants of FOR16D, and indicates the ESTs that have been utilised to determine the open reading frames of the introns that collectively provide for the alternate splice variants of FOR16D transcripts. Also shown are the sequence positions of the exons shown from the position of the respective EST from which sequence was obtained.

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- Figure 7 Is a second diagrammatic representation of FOR16D alternate transcripts with respect to FRA16D and common homozygous deletions. A. and B. are duplications of Figure 1 (above). C moving from top to bottom shows to relative position of certain mutations relative to a restriction enzyme map of the YAC My801B6, as well as the relative location of two further YACs My891F3 and My972D3. Below that are shown the position of four BACs, below that are shown the position of deletions in the cell lines AGS and HCT116, also shown is the position of the c-MAF oncogene. Below that are shown the regions that are sequenced, and the location of three multiple myeloma translocation break points. Following that are shown the four known alternate spliced transcripts and a listing of the EST that confirm the position of the four transcripts.
- Figure 8 is a diagrammatic representation of four of the predicted splice variant transcripts as well as representation of a Northern blot analysis of RNA from various physical locations indicated using a portion of exon 3 as a probe. A similar result is found when a probe from exon X is used.
- Figure 9 is a composite DNA sequence of the predicted FOR16DI transcript. The composite has been constructed by conjoining ESTs as indicated.
- Figure 10 is a composite DNA sequence of the predicted FOR16DII transcript. The composite has been constructed by conjoining ESTs as indicated.
- Figure 11 is a composite DNA sequence of the predicted FOR16DIII transcript. The composite has been constructed by conjoining ESTs as indicated.
- Figure 12 is a composite DNA sequence of the predicted FOR16DIV transcript. The composite has been constructed by conjoining ESTs as indicated.

- Figure 13 are composite amino acid sequences predicted for the sequences for FOR16DI, FOR16DII, FOR16DIII and FOR16DIV as shown in figures 9 to 12.
- 5 Figure 14 sets out certain amino acid homologies of the predicted amino acid sequence for FOR16DIV and FOR16DI, using the Blast program (Altschul *et al* (1997) *Nucleic Acids Res.* 25:3389-3401) and the swissprot database. Each comparison sets out the swiss prot number assigned to the sequence compared with, the FOR16D amino acid sequence is on top (:) indicates sequence identity and (+) indicated conserved substitution the bottom sequence of each comparison is the sequence accessed from the swissprot database.
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- Figure 15 sets out DNA sequences for each of the exons identified for the FOR16D protein.
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- Figure 16 is about 270kb of DNA sequence that overlaps and defines within it the FRA16D fragile site, which is shown to reside between exons 4 and 5.
- 20 Figure 17 is DNA sequence for contig #208 as indicated in figure 6, and which encompasses exon 3,
- Figure 18 is DNA sequence for contig #779 as indicated in figure 6, and which encompasses exon 2,
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DETAILED DESCRIPTION OF THE INVENTION.

EXAMPLE 1 - MAPPING OF THE FRA16D FRAGILE SITE

30 Materials and methods

Isolation of DNA probes and YACs in the FRA16D region

Nine DNA probes, ACH202 (D16S14), c311F2, c302A6 (D16S1075), c301F10 (D16S373), 16-87 (D16S181), c306D2, 16-08 (D16S162), c307A12 and CRI-0119 (D16S50) which had been physically mapped into the 16q23 region (30) were chosen for fluorescence *in situ* hybridisation (FISH) against *FRA16D* expressing chromosomes. Four of these markers mapped within the same somatic cell hybrid breakpoint interval defined by the cell lines CY113(P) and CY121 (30). One of these, c306D2 mapped proximal to *FRA16D* by FISH while the others, c307A12, CRI-0119 and 16-08 mapped distal to *FRA16D*. These probes were therefore used as starting

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points to isolate a contig of cloned DNA spanning *FRA16D*. In the Los Alamos National Laboratory database (www-ls.lanl.gov) an STS sequence from c306D2 was found within the CEPH YACs My903D9, My912D2 and My933H2 while an STS in c307A12 was found in My891F3 and My972D3. These YACs were obtained from CEPH and the prepared DNA subjected to *Pst* I digestion, Southern blotted and probed with 16-08, 16-87, CRI-0119, c306D2 and c307A12 in succession in order to confirm their content. In addition a search of the Whitehead Institute database (www-genome.wi.mit.edu) revealed that the two sets of YACs were joined into a contig by the YACs My801B6, My845D9 and My944D8. Each of these YACs was used as template DNA to assess STS content (D16S518, Afma336yg9, WI2755, STSG-10102 and D16S3029) and subjected to FISH to assess position with respect to *FRA16D* (Figure 1B).

Additional probes, STSs and BACs from the FRA16D region

Additional probes were generated from the YAC 801B6 by subcloning *Pst* I digests of YAC DNA and screening with total human DNA as probe. These subclones were digested with *Hinc* II to identify and isolate non-repetitive DNA fragments as probes. This generated markers H13m, H22s, H23m, H29m and H40m. Genome System Inc. BAC library filters were screened with the probes D16S518, Afma336yg9, WI-2755, STSG-10102, H22s, H29M and D16S3029 and nine BAC clones including 379C2, 325M3 and 353B15 were identified. An additional STS, named 2AS, was established by 'bubble' PCR from the end-fragment of BAC 353B15 and was isolated as described by Gecz *et al* (31). Briefly, the BAC DNA was digested with *Alu* I and ligated to the annealed bubble linkers. The final PCR was carried out with a combination of *Not* I-A bubble primer and Sp6-promoter primer as described except an annealing temperature of 55°C was used. These STSs and hybridisation probes were used to establish restriction maps of the YAC My801B6 and the BACs (Figure 2A).

Subcloning and contig assembly

The YAC My801B6 and the BAC 325M3 were used as DNA templates for establishing a lambda subclone libraries in λGEM11 or λGEM12 vectors (Promega) according to the supplier's protocol. My801B6 and 325M3 appeared to have intact human DNA inserts, based on comparative pulsed field gel mapping of the YACs and BACs across the region (data not shown).

Fluorescence in situ hybridisation

FRA16D-expressing metaphases were obtained from peripheral blood lymphocytes by standard methods. Briefly, cultures were grown for 72 hours in Eagle's minimal essential medium, minus folic acid, supplemented with 5% fetal calf serum.

Induction of *FRA16D* was with 0.5uM aphidicolin (dissolved in 70% ethanol) added 24 hours before harvest (32). DNA clones were nick-translated with biotin-14-dATP, pre-associated with 6ug/ul total human DNA, hybridised at 20ng/ul to metaphase preparations, and detected with one or two amplification steps using biotinylated anti-avidin and avidin-FITC as previously described (33). Hybridisation signal was visualised using an Olympus AX70 microscope fitted with single pass filters for DAPI (for chromosome identification), propidium iodide (as counterstain) and FITC. *FRA16D*-expressing chromosomes were scored for signal only when the width of the fragile site gap was greater than the width of one chromatid, so that signal was unambiguously proximal or distal to the gap (Figure 3). Only fluorescent dots which touched chromatin were scored as signal - the few fluorescent dots which lay within the fragile site gap but did not touch proximal or distal segments were therefore not scored as signal since there was a possibility that they comprised non-specific background. Lambda clones which gave very poor FISH results (high non-specific hybridisation to other chromosomes) were not able to be scored with respect to the fragile site. This is likely to be due to the large amount of repetitive DNA within these particular clones - see below.

Tumour cell lines

The tumour cell lines LoVo, HT29, Kato III, SW480, AGS, MDA-MB436 and LS180 were purchased from the American Type Culture Collection. LoVo and AGS cells were grown in Hams F12 medium with 2mM L-glutamine, 10% fetal calf serum in 5% CO₂, Kato III cells were grown in RPMI1640 medium with 2mM L-glutamine, 20% fetal calf serum in 5% CO₂, HT29 cells were grown in McCoy's 5a medium with 1.5mM L-glutamine, 10% fetal calf serum in 5% CO₂, LS180 cells were grown in Eagle's minimal essential medium with 2mM L-glutamine and Earle's salts and non-essential amino acids, 10% fetal calf serum in 5% CO₂, SW480 cells were grown in Leibovitz's L15 medium with 2mM L-glutamine and 10% fetal calf serum, MDA-MB-436 cells were grown in Leibovitz's L15 with 16µg/ml glutathione and 0.026units/ml insulin.

PCR detection of homozygous deletion in tumour cell DNAs

PCRs for the detection of individual sequence tagged sites from across the *FRA16D* region were duplexed (34) with control PCRs from the dystrophin gene on the X chromosome (DMD Pm or DMD49, ref 35) or the APRT gene on chromosome 16 (33). This allowed verification that the PCR reaction was working in the absence of a *FRA16D* region PCR product (Figure 4). Suitable PCR primers for Alu29, 17Sp6, Alu20, 178poly, 5.1A6, RD69, IM7 were used or for 504CA, forward 5'-AACACAGCTCTTATCACATCC- 3', reverse 5'-TGGCTGTAmGTCAGAACTG- 3';

while others were as given in database accessions, D16S518 (GenBank Z24645), Afma336yg9 (GDB 1222843), WI2755 (GenBank G03520), STSG-10102 (GenBank Z23147), D16S3029 (GDB 605884), WI-17074 (G22903), IM9 (GenBank R05832), D16S3096 (GenBank), D16S516 (GDB 200080). PCR for GenBank AA368108 (forward 5'-TAATCCTCAGCCTCTAGAATGCCT-3', reverse 5'-GTATGATGATTTTCAGGGAGAAAC-3') and GenBank AA398024 (forward 5'-TGTCCTCAACTGATTCTTACAAAC-3, reverse 5'-TCAATGGGTTAGGCACAGACC-3') were derived from partial sequence analysis of BAC353B15. Control PCRs for FRA3B deletions were D3S1234 (GDB 186387), D3S1300 (GDB 188420) and D3S1841 (GDB 254090).

Results

Positional cloning of FRA16D

A contig of YAC clones was established in the 16q23.2 region between markers c306D2 and c307A12 which were found by FISH to map proximal and distal to *FRA16D*, respectively (Figure 1B). The individual YACs from this contig were also used as hybridisation probes to further localise the fragile site. These experiments identified the YAC 801B6 as spanning *FRA16D*, and therefore this YAC was used as a source of DNA for subcloning the region to provide shorter DNA fragments for further refinement of the fragile site position. In addition, BAC clones were identified from the region to provide redundancy of cloned human DNA in an effort to avoid potential problems of instability of human DNA in YACs, as has previously been noted for other fragile site regions, including *FRAXA* (37), *FRA10B* (38 and O. Handt, *pers. comm.*) and a Chinese hamster aphidicolin inducible fragile site region (39).

A pulsed-field gel restriction map of YAC 801B6 was constructed by using *HincII* restriction fragment subclones of the YAC for use as hybridisation probes (H13m, H22s, H23m, H29m and H40m) (Figure 2A). The position of the BACs (379C2, 325M3 and 353B15) with respect to the YAC restriction map was determined by both the restriction mapping of the BACs and the positioning of common markers by PCR or hybridisation (Figure 2A). The STS (D16S518, Afma336yg9, WI2755, STSG-10102 and D16S3029) content of the YACs and BACs was also determined to assist in map construction.

Subclone libraries of DNA from YAC 801B6 and BAC 325M3 were generated using the lambda vectors λ GEM12 and λ GEM11 (Promega), respectively and assembled into a contig by end-fragment hybridisation and restriction mapping. The integrity of the YAC restriction map was verified by comparison with that of the BACs, 325M3 and

353B15. For the region between the BACs the integrity was verified by the use of long range PCR using human chromosomal DNA as template. (data not shown).

Localisation of FRA16D by fluorescence in situ hybridisation (FISH)

5 There have been difficulties in determining the precise localisation of common chromosomal fragile sites using FISH (refs *FRA3B* (13, 40,41,42), *FRA7G* (18,19) and *FRA7H* (43). The FISH data have been interpreted as due to the fragile sites being spread out over long DNA sequences (eg 100's of kb) or that there are multiple fragile sites at a single locus. An alternative explanation is that the DNA in the immediate
10 vicinity of the fragile site is not tightly 'packaged' into chromatin. We therefore chose to score only those chromosomes where the width of the gap or break at the *FRA16D* fragile site was greater than that of one chromatid (Figure 3). This approach was intended to reduce the possibility that the 'unpacked fragile site DNA' might be looping back over the distant side of the fragile site and therefore give a false
15 'spanning' signal - particularly for probes that are very close to or within the fragile site region. In addition, while the use of pre-reassociation in the hybridisation process dramatically improved the signal to noise ratio, it did render repeat rich regions poor hybridisation probes. This was particularly evident in the *FRA16D* region where there is an abundance of DNA repeat sequences of various kinds.

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The results of the FISH experiments are plotted in figure 4. The closest clearly proximal probe to *FRA16D* is λ 1-44 while the closest unequivocally distal probe is λ 433. These probes map at a distance of ~200kb apart. However, this 200kb region includes consistent scatter of distal signal around λ 1-38 and λ 1-27 and the poor
25 hybridisation between λ 181 and λ 511 (due to repetitive DNA content). Therefore this 200kb defined by FISH analysis is likely to be the maximum sequence required to define *FRA16D* rather than provide any evidence that the fragile site is spread over such a distance.

30 *Detection of homozygous deletion in tumour cell lines*

The *FRA3B* fragile site - FHIT gene intron 4 region is a frequent site of deletion in various types of cancer (8). Homozygous *FRA3B* deletions have been detected in various human adenocarcinoma cell lines including (gastric) AGS, Kato III; (breast) MDA-MB436; (colon) LoVo, HT29, SW480 and LS180 (8). Since these deletions are
35 somatic events that presumably occur as a result of exposure of these cells to certain environmental factors (11), we chose to analyse tumour cell lines which exhibit *FRA3B* deletions for the presence of homozygous deletion at the *FRA16D* locus.

STSs that were either mapped to the *FRA16D* region (Figure 1) or generated from partial sequence analysis through the region (data not shown) were used to screen for homozygous deletion in various tumour cell line DNAs. The STSs were duplexed with a PCR from the dystrophin locus, as an internal control. The results for the analysis of one of the *FRA16D* region markers, STSG-10102 is shown in figure 4. Of the seven tumour cell lines tested, the stomach tumour cell line AGS was found to be homozygously deleted at STSG-10102 and a series of contiguous markers through the region, (Table 1) thus suggesting the presence of minimal deletions spanning the *FRA16D* region in each chromosome 16 present in the AGS cell line.

Detection of heterozygous deletion in AGS tumour cell line DNA

The maximal extent of heterozygous deletion in the AGS tumour cell line in the *FRA16D* region was determined by genotyping polymorphic markers. The markers D16S518 and D16S3029 both gave two alleles indicating proximal and distal outer limits to the deletion of either chromosome 16 in AGS cells (Figure 2A). The markers Afma336yg9 and 504CA were uninformative and therefore did not aid in delineating the limits of heterozygous deletion.

Discussion

The region in which the chromosomal fragile site *FRA16D* is located has recently been shown to be associated with two types of chromosomal instability in cancer. In multiple myeloma, translocation of Ig loci into the 16q23 region causes the dysregulation of the *c-MAF* proto-oncogene on the affected allele. While these breakpoints are spread over at least 500kb they bracket both the *c-MAF* gene and the *FRA16D* fragile site (1 and figure 1). The dysregulated expression results in elevated *c-MAF* mRNA levels, which is thought to contribute to neoplasia. These translocations were not identified by conventional cytogenetic analysis. Their detected frequency in multiple myeloma cell lines suggests an incidence of ~25%.

Using representational difference analysis to identify differences between the genomes of normal and tumour cells, the *FRA16D* region has also been shown to be the site of homozygous deletion in three different types (lung, ovary and colon) of adenocarcinoma (29). The commonly deleted region includes *FRA16D*, with the minimal deletion in colon tumour cell line corresponding almost exactly to the ~200kb region shown by our FISH studies to span the *FRA16D* fragile site. If common aphidicolin fragile sites confer susceptibility to mutagen induced DNA instability in cancer then tumour cell lines which have been shown to have such instability at one fragile site are likely to exhibit instability at another fragile site. By analysing tumour cell lines with known *FRA3B* deletions, we have found that the AGS cell line derived

from a stomach cancer exhibits homozygous deletion spanning *FRA16D*. Heterozygosity of the flanking markers D16S518 and D16S3029 indicates that the chromosome 16 deletions are confined to the immediate vicinity of *FRA16D*.

- 5 Taken together these deletion data confirm the hypothesis that *FRA16D* is associated with specific chromosomal instability in cancer.

Given that the observed deletions are homozygous they are therefore likely to represent the loss of a negative function (eg tumour suppressor) rather than the gain of a tumour promoting function. If the analogy with the *FRA3B* locus holds then a gene either spanning or, at least partially, within the *FRA16D* commonly deleted region may contribute to neoplasia as a consequence of quantitative and/or qualitative effects of the deletion. Alternatively, the proximity of the *FRA16D* deletions to the *c-MAF* gene suggests that they have the potential to affect *c-MAF* expression. The *FRA3B* fragile site is associated with a region of 'late' replication (48) as are the 'rare' fragile sites *FRAXA* and *FRAXE* (49,50). Assuming that replication timing is affected by proximity to fragile site loci and, given the coupling of replication with transcription, the deletion of the *FRA16D* region may lead to an alteration in the timing, with respect to the cell cycle, of the expression of genes in the area - including *c-MAF*.

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ABBREVIATIONS BAC, bacterial artificial chromosome; DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridisation; FITC, fluorescein isothiocyanate; LOH, loss of heterozygosity; FHIT, fragile histidine triad; FRA, fragile site locus; PCR, polymerase chain reaction; STS, sequenced tagged site; YAC, yeast

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EXAMPLE 2 - DNA SEQUENCING OF THE *FRA16D* FRAGILE SITE AND THE *FOR16D* GENE.

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Materials and Methods

Large scale sequencing of FRA16D included

- a) Sonication libraries and
- b) Nebulization libraries of BAC clones 325M3 and 353B15 and
- c) Restriction fragments of Lambda clones

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(for sequencing between BAC325M3 and BAC353B15)

- a) Construction of sonication libraries:

10µg of each BAC DNA were sonicated for 20 seconds using the Ultrasonic Inc. Heat Systems Sonicator (50% duty, 3.5 power).

Blunt ends were created with 40 U of Mung Bean Nucleases at 30 °C for 25 minutes. The products were size fractionated on a 1% Agarose gel and fragments ranging from 1.9-0.8 kb were extracted from the gel with the Qiaquick Gel Extraction Kit.

1500 ng of sonicated DNA were ligated into pUC-Sma plasmid vector and cloned into
5 Sure cells (electroporation-competent, Stratagene).

600/1500 clones of the sonication libraries of BAC 325M3/353B15 respectively were gridded on 96 well plates and sequenced in one direction using the M13-forward primer.

10 Sequences were assembled into contigs in the gap4-program on an UNIX computer. For a selected number of clones sequences with the M13-reverse primer were also retrieved and assembled. Restriction maps of the contigs were compared to physical mapping data. Rearranging and editing of the sequence was undertaken with the "LaserGene" computer program.

15 Numerous primers were designed and PCR-products sequenced to close gaps between contigs.

b) Construction of nebulization libraries:

10 µg of each BAC DNA were nebulized at 10psi for 45 seconds.

20 Size-fractioning and cloning was done as described above.

300/500 clones of BAC 325M3/353B15 respectively were sequenced as described above and included in the assemblies.

Subclones for sequencing of BAC353B15 were picked randomly, whereas
25 BAC325M3 subclones were selected after specific hybridisation experiments.

c) Subcloning of restriction fragments of selected λ-clones was done in pUC19-vector. Clones were sequenced with M13-forward+reverse primers as well as with specific primers.

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The Nucleic Acid Sequence (FOR16D)

The inventors have prepared a DNA sequence for the FRA16D fragile site and the minimal overlapping region of homozygous deletion in adenocarcinomas of the lung, colon, stomach and ovary and in doing so have discovered a gene located at
35 chromosome 16q23.2 and determined its DNA sequence.

An overview of the sequence data can be seen in Figures 6 and 7. An approximate restriction enzyme map is shown in figure 7c was prepared for the YAC My801B6.

Sequence for FRA16D was obtained primarily from BAC 353B15. The DNA sequence of FRA16D is presented in figure 16. This is approximately 270kb long and is bounded at both ends by an exon (termed exon 4 and exon 5 respectively).

- 5 The exons were compared with ESTs in the GENBANK data base and two EST clusters were identified. These are indicated as I and II respectively in Figure 7. Both of these are splice variants of the one gene. Further sequence data for contigs #208 and contig #779 was obtained from BAC 325M3 to identify two further exons 2 and 3 respectively. The DNA sequence of contigs #208 and contig #779 are presented in
 10 figures 15 and 16 respectively. Homologies for the unlocalised DNA was searched for again in the same database and this identified a further EST cluster, termed ESTIII which. The EST's with homologies are again listed in figure 7. DNA sequence information of the BAC 009280 identified a further exon which was termed exon z. The remainder of the unlocalised portion of the EST cluster was termed exon a. A
 15 further exon was identified on searching through EST databases for homologies with exon a to identify a yet further EST cluster ESTIV which is a combination of exons a and w.

- The sequence defining the FRA16D site is flanked by two exons of the *FOR16DI* gene
 20 with no other detected transcript within this intron. In addition, the breakpoints of three out of five 16q23.2 translocations associated with multiple myeloma (Chesi *et al* 1998) map within the alternate splice of this *FRA16D* intron, that is between exons 4 and x.

- DNA sequence for each of the exons was compiled by a comparison of the EST
 25 clusters against each other as well as against chromosomal sequence. These are set out in Figure 15.

- The position of exons a, 1, z, w 6 and x have only been approximately mapped on the basis of their presence on certain subclones (containing localised markers within
 30 16q23) as judged by hybridisation experiments.

- Composite DNA transcript sequences have been prepared of the EST clusters and putative DNA sequences for four variants of the gene *FOR16D* (I, II, III and IV) have been compiled and are presented respectively in figures 9, 10, 11, and 12.

- 35 The predicted amino acid sequence are presented in figures 13A to D. These amino acid sequences were compared with amino acid sequences stored on the Swissprot amino acid sequence database using the program BLAST (Altschul *et al* (1997) *Nucleic Acids Res.* 25:3389-3402). Two significant group of homologies were found. A first

of the homologies is identified in FOR16DIV relative to a protein related to DNA replication (HumanPeptidyl-prolyl cis tran isomerase and three other proteins. As can be seen the string LPPGWEERT appears highly conserved. This string lies within exon A and implicates that exon as having a role in DNA replication or Chromosomal division. This is compatible with the FOR16D being associated with tumourigenesis in that one group of proteins having an association with cancer fall into this group. This amino acid string and the DNA sequence encoding may also be very useful for identifying other cancer associated genes.

Another group of homologies that were found are further downstream for the FOR16DI gene these provide or several relatively strong homologies in several different amino acid strings for some oxidoreductase genes. These strings include VVVVTGANSIG, MTLDLALLRSVQ, PLDVLCNAA and VNH LGHFYL. There is a potential that FOR16D has an oxidoreductase activity. Association of oxidoreductase activity with a protein associated with DNA replication or chromosomal division has to date not been published.

The RNA Transcript

The estimated size of the major alternatively spliced transcript, as determined by Northern blotting (Figure 8) using a portion of exon 3 as probe, is about 2400 nucleotides and most likely corresponds to the transcript of FOR16DII, there is a smaller transcript which is about 1.6kb and this is most likely to correspond to transcript I. A similar experiment has been conducted where the probe is selected from exon x and the 2.4 kb transcript is seen again, supporting the view that the 2.4kb transcript is the FOR16DI transcript.

For the purposes of working the invention a large number of references to pertinent methodologies are set forth in the following US patent documents:- US 5981218 to Rio *et al*, US 5928884 to Croce *et al*, US 5945522 to Cohen *et al*, and US 5837492 to Tavtigian *et al*. These documents are incorporated herein entirely specifically for purposes of permitting working of the invention.

For the purposes of this specification the word "comprising" means "including but not limited to", and the word "comprises" has a corresponding meaning.

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- 45 Dated this 16th day of December 1999

Figure 1:
Positional cloning of *FRA16D* and location of loss of heterozygosity and translocation in cancer

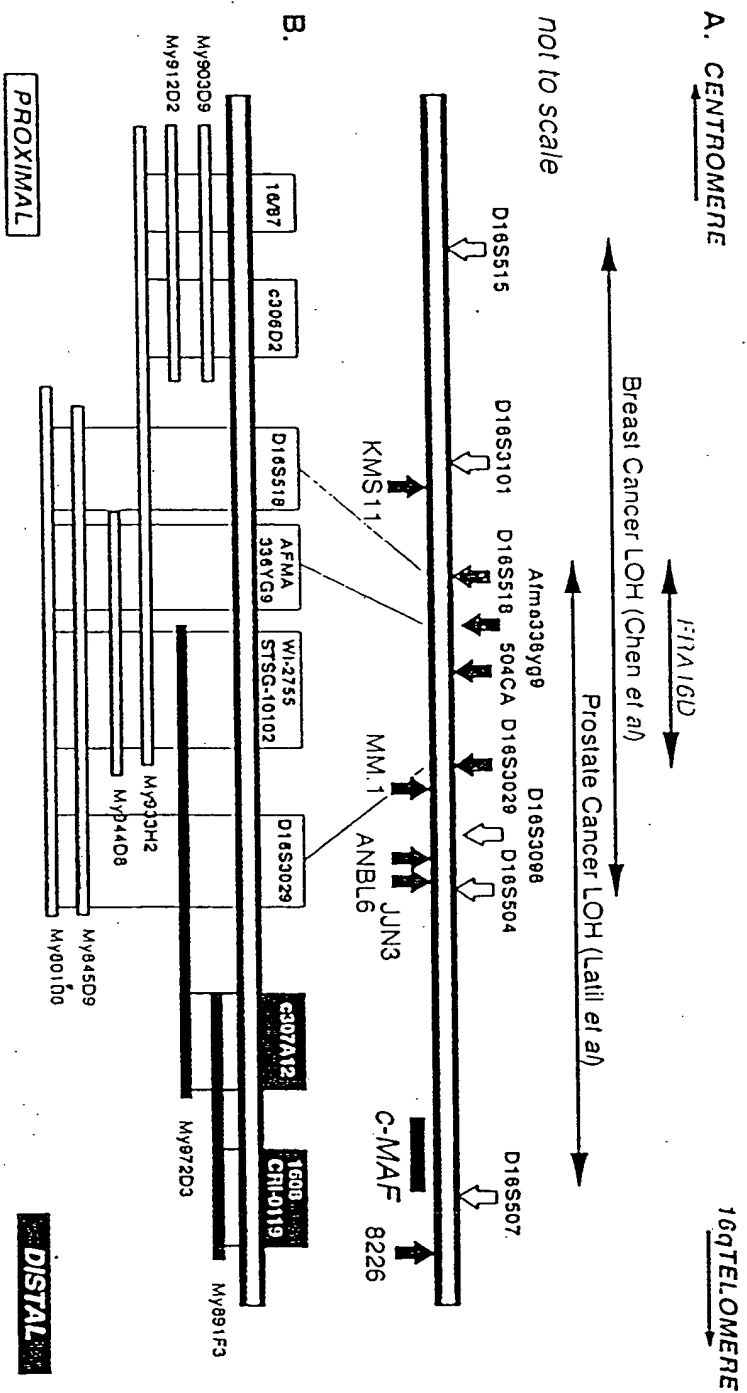


Figure 2:
Positional cloning of *FRA16D* and the extent of heterozygous and homozygous deletion in the AGS tumour cell line.

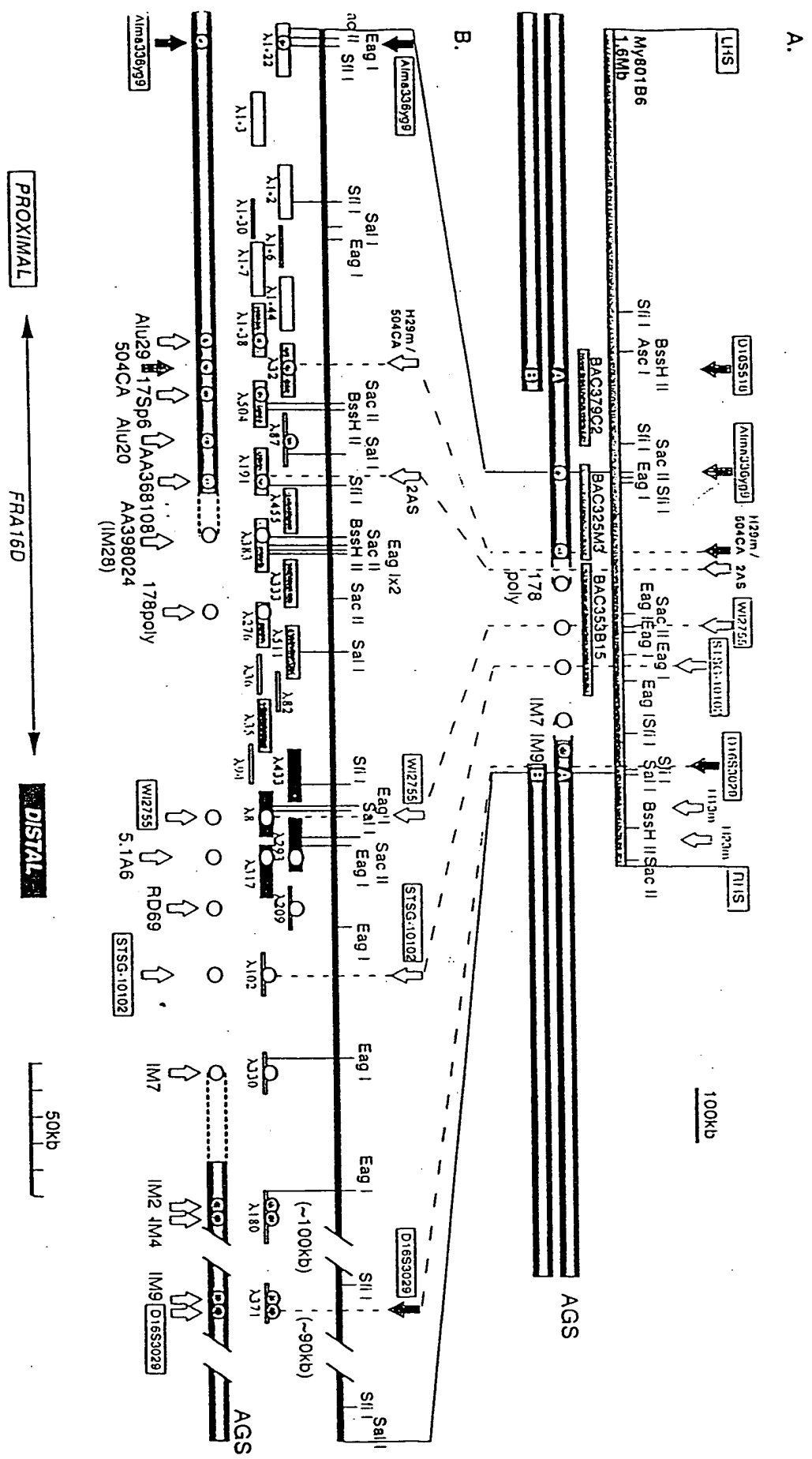


Figure 3: Fluorescence *in situ* hybridisation (FISH) of lambda subclones against *FRA16D* expressing chromosomes

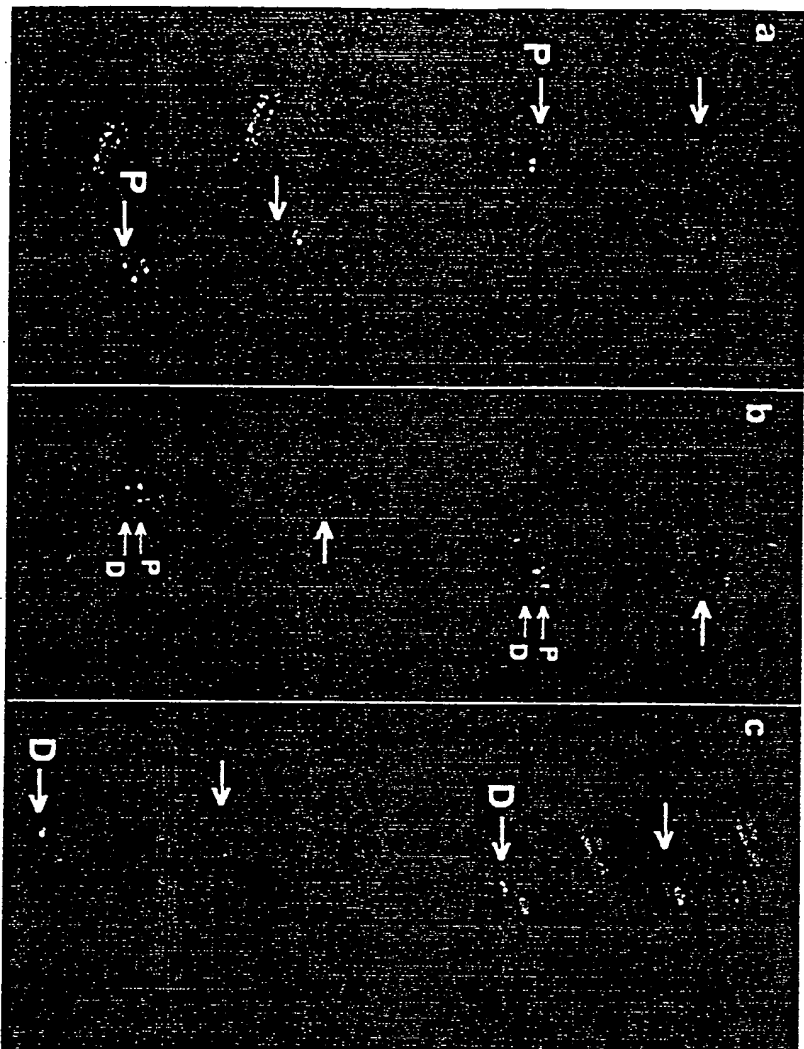


Figure 4: Fluorescence *in situ* hybridisation of lambda subclone contig to FRA16D

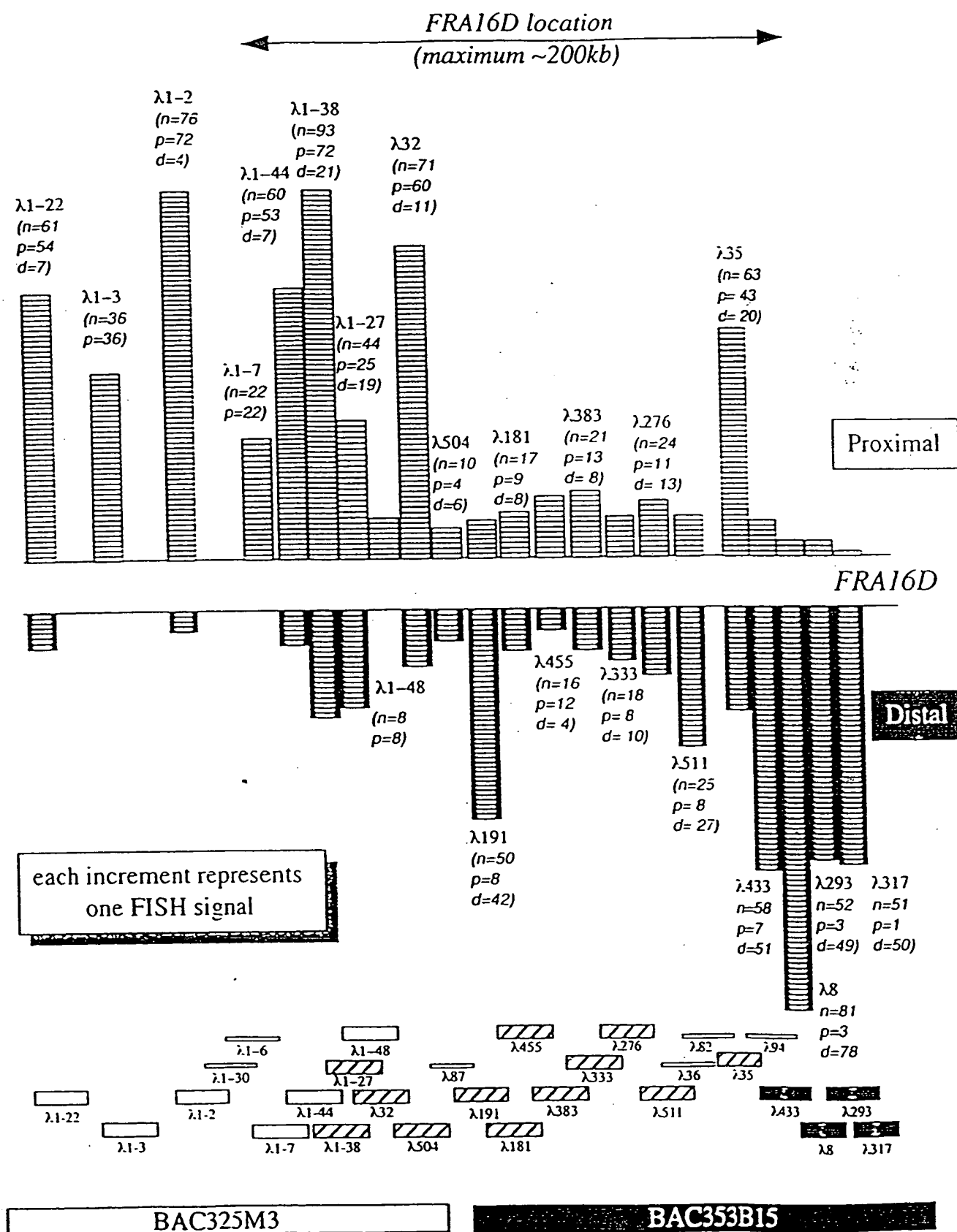
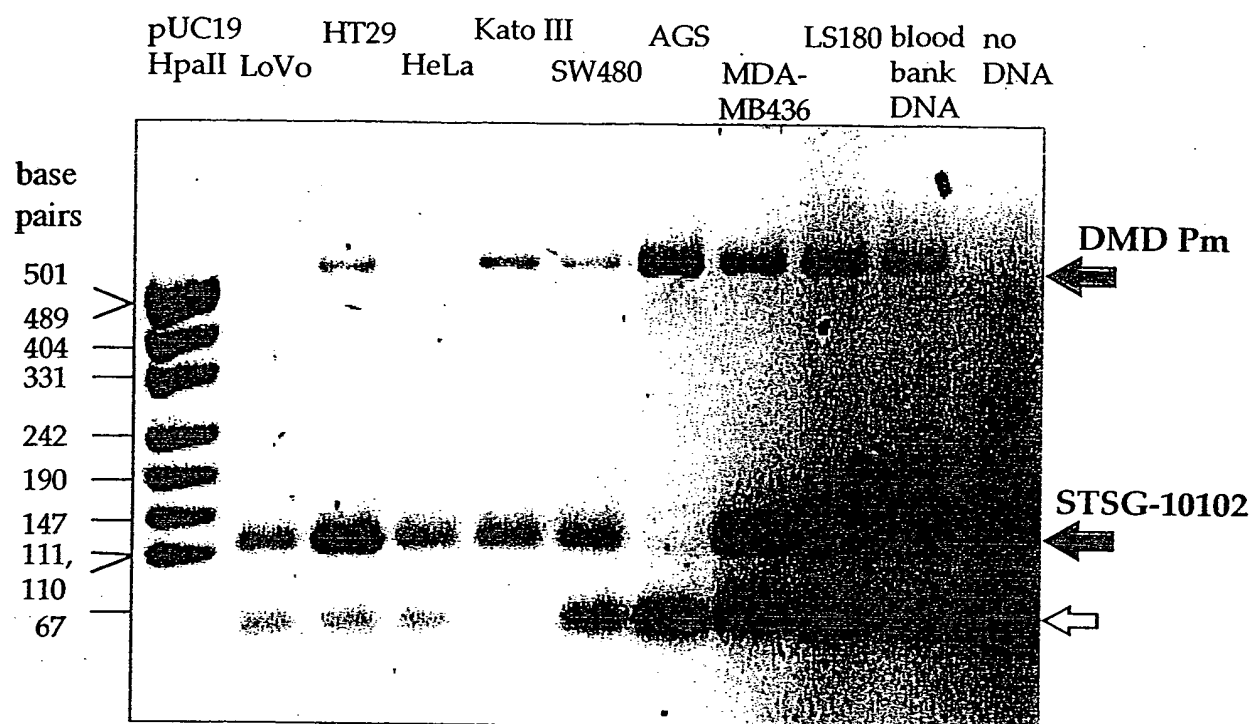
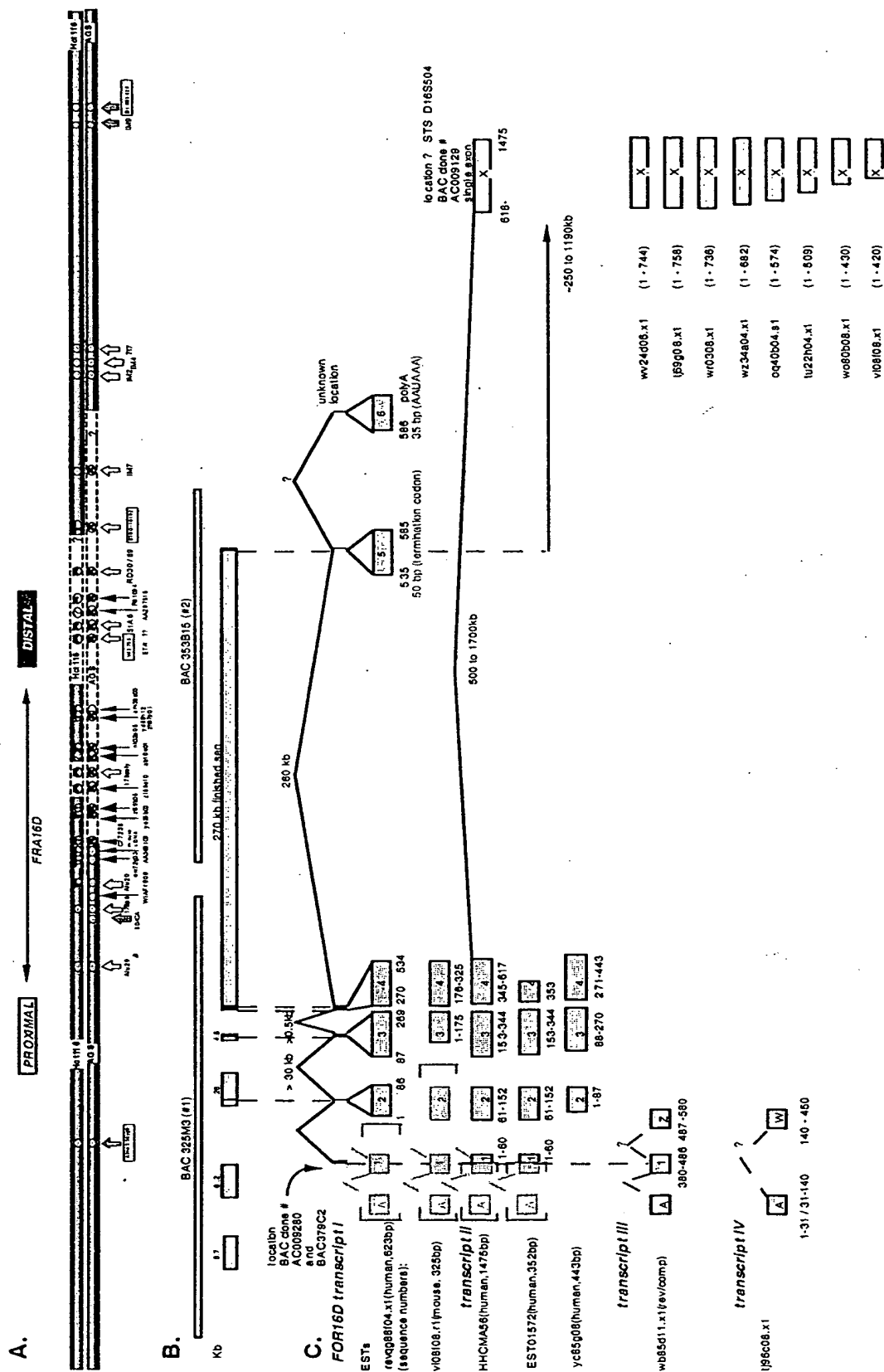


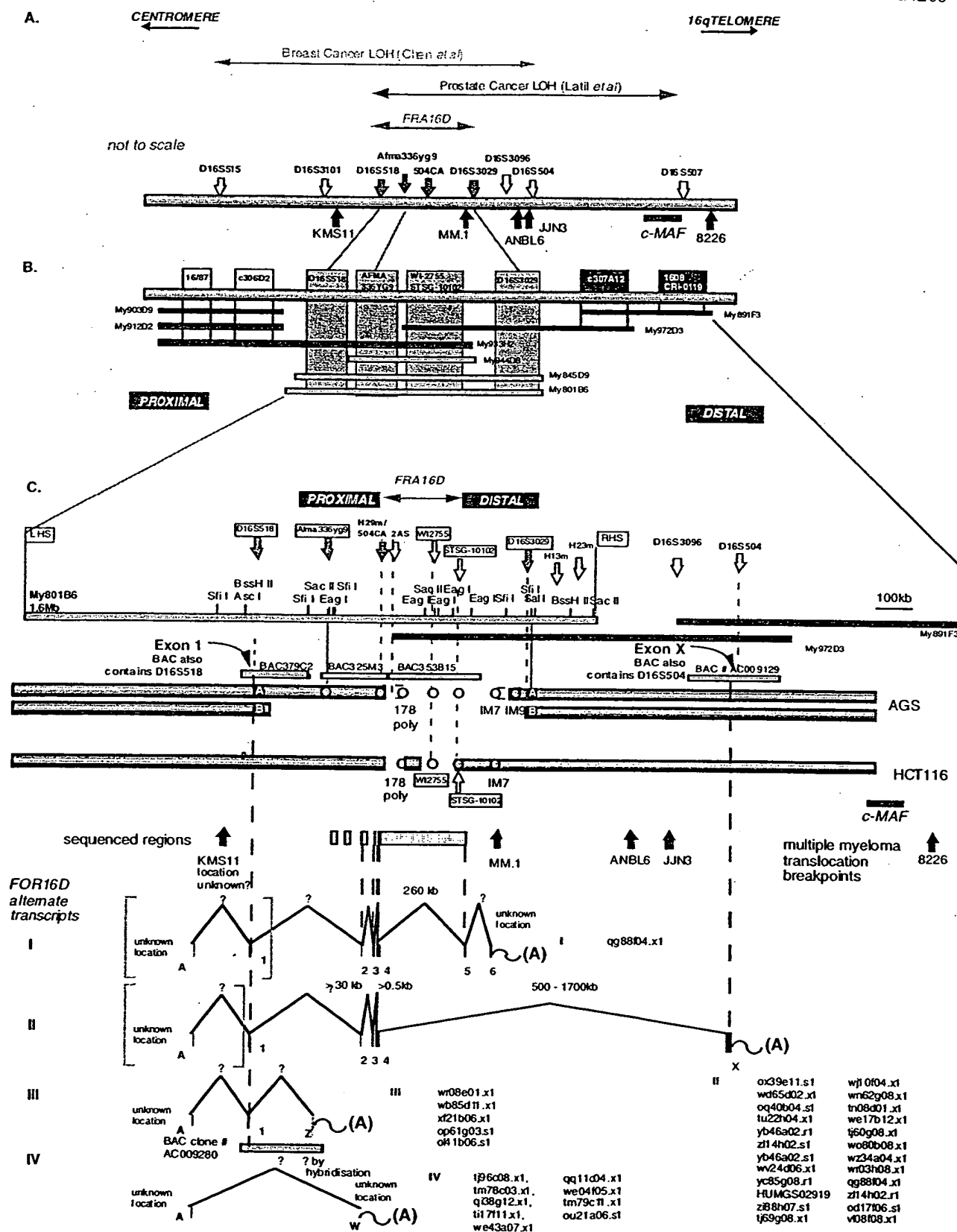
Figure 5 : Duplex PCR deletion detection at the *FRA16D* locus in tumour cell lines



Map of *FOR16D* transcript with respect to *FRA16D* and common homozygous deletion



16/12/99



FOR16D alternatively spliced transcripts

FIGURE 8

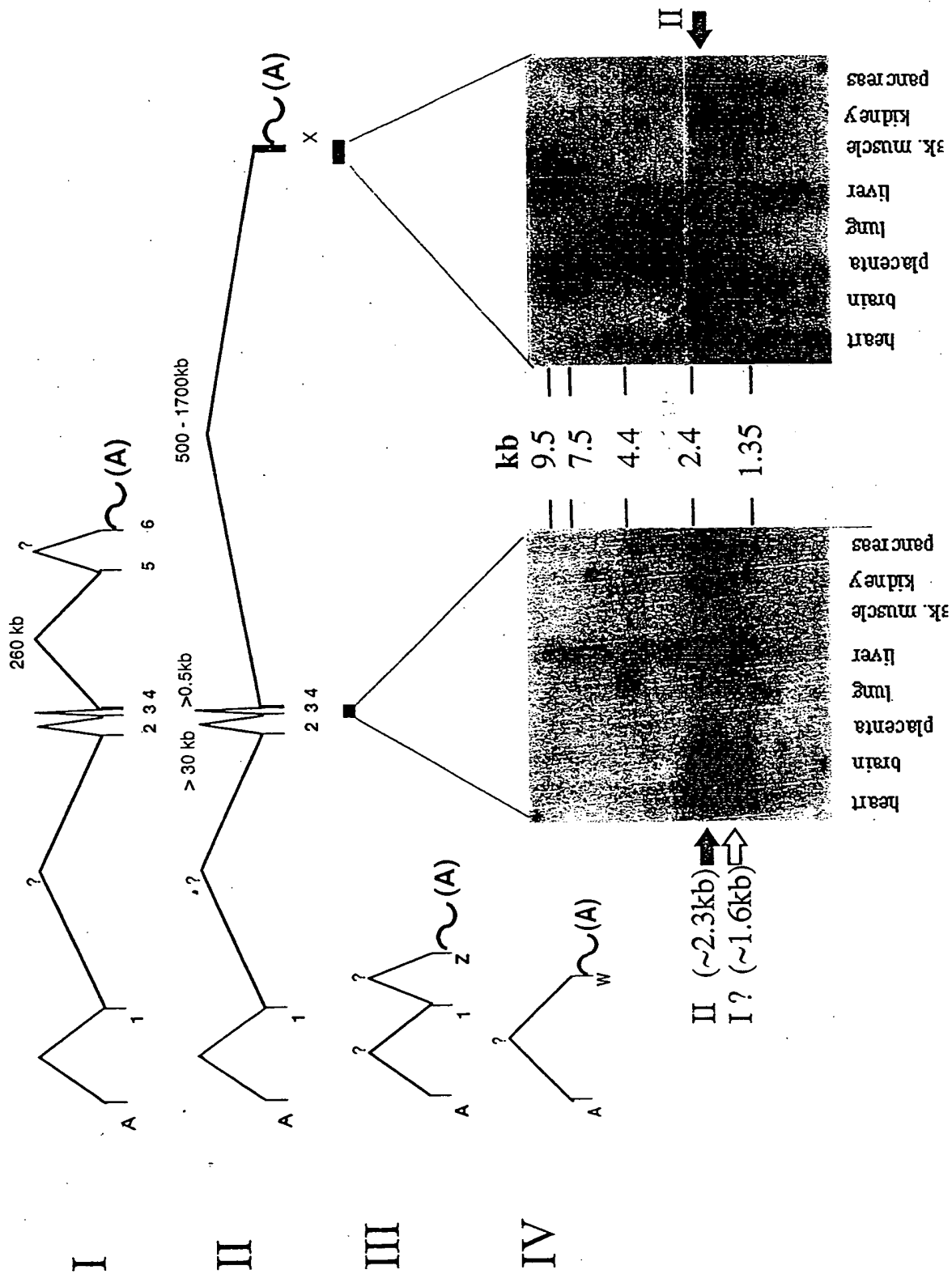


FIGURE 9

Composite FOR16DI transcript

tj96c08.x1, wb85d11.x1, HHCMA56, qg88f04

GCGTAGGGGggccaggtgcctccacagtcaagccATGgcagcgctgcgctacccggggctg
gacgacacggacagtgaggacgagctgctccggggctgggaggagagaaccacaaaggac
ggctgggtttactacgccaatcacaccgaggagaagactcagtgggaacatccaaaaact
ggaaaaagaaaacgagtggcaggagatttgccatacggatgggaacaagaaactgatgag
aacggacaagtgtttttgttgaccatataaataaaagaaccacctacttgacccaaga
ctggcggtttactgtggatgataatccgaccaagccaaccacccggcaaagatacgacggc
agcaccactgccatggaaattctccagggccgggatttcactggcaaagtgcgttgtggtc
actggagctaattcaggaataggggttcgaaaccgccaagtcttttggcctccatggtgca
catgtgatcttggcctgcaggaacatggcaagggcgagtgaaagcagtcacgcattt
gaagaatggatcttggcctgcaggaacatggcaagggcgagtgaaagcagtcacgcattt
tagaagaatggcatagggccaaggtagaaacaatgaccctggacctcgctctgctccgt
agcgtgcagcattnctgtgaagcattcaaggccaagaatgtgcctcttcagtgcttggtg
tgcaacgcagcaacttttgctctacccggaggtctcacaagatggcctggagacaccttc
caagtgaatcatctggggcactttctaccttgtccagctcctccaggatgttttgtgcgc
tcagctcctgcccgtgtcatttgtggtctcctcagagtcctcatcgatttacagatattaac
gactccttgggaaaactggacttcagtcgcctctctccaacaaaaaacgactattggggc
atgctggcttataacaggtccaagctctgcaacatcctcttctccaacagagctgcaccgt
cgctctccccacgcggggtcacgtcgaacgcagtgcatcctggaaatatgatgtactcc
aacattcatcgagctgggtgggtgtacacactgctgtttaccttggcgaggcctttcacc
aagtccatgggttcagactgcctggtagaaggaggtcacttctgattgtcagtgactttg
agctgagtgctgaaataaaatgataaacaagtcaaaaa

FIGURE 10

Composite FOR16DII transcript

tj96c08.x1, wb85d11.x1, HHCMA56

GCGTAGGGGgcccaggtgcctccacagtcagccATGgcagcgctgcctacccgggctg
gacgacacggacagtgaggacgagctgctccggggctgggaggagagaaccacaaaggac
ggctgggtttactacgccaatcacaccgaggagaagactcagtggaacatccaaaaact
ggaaaaagaaaacgagtggcaggagatttgccatacggatgggaacaagaaactgatgag
aacggacaagtgtttttgttgaccatataaataaaagaaccacctacttcgacccaaga
ctggcgtttactgtggatgataatccgaccaagccaaccacccggcgaagatacgacggc
agcaccactgccatggaaattctccagggccgggatttcactggcaaagtgcgttgtggtc
actggagctaattcaggaatagggttcgaaaccgccaagtcttttgccctccatgggtgca
catgtgatcttgccctgcaggaacatggcaagggcgagtgaaagcagtggtcacgcatttta
gaagaatggatcttggcctgcaggaacatggcaagggcgagtgaaagcagtggtcacgcatt
ttagaagaatggcataaaagccaaggtagaagcaatgacctggacctgccttcgctccg
agcgtgcagcatttttgctgaagcattcaaggccaagaatgtgcctcttcagtcgcttcg
tgcaacgcagcaacttttctctaccctggagcttcaccaagatggccttcagaccacc
tttcaagtgaatcatctggggcacttctaccttggtccagctcctccagggatgtttgt
ggcgtcagctcctgcccgtgtcatttgtggtctcctcagagtcccatcgatttacagata
ttaacgactccttgggaaaactggacttcagtcgcctctctccaacaaaaaacgactatt
ggcgatgctggccttataacaggtccaagctctgcaacatcctcttctccaaagagctgc
accgtcgcctctccacgcggggtcacgtcgaaagcagtcgatcgatcctggaaaatagat
gtactccaacattcatcgagctgggtgggtgtacacactgctgtttaccttcggagggc
tttcaccaagtcctatgcaacagggagctgccaccaccgtgtactgtcctgcctgcccaga
actggagggctcaggagggatgtacttcaacaactgctgccgctgcacccctcaccaga
agctcagagcgaagagacggcccgaccctgtgggcctcagcgagagggctgatccaagaa
cgcttggcagccagtcgggctaagtggagctcagagcggatgggcacacacacccgccct
gtgtgtgtccctcacgcaagtgccaggctgggccccttccaaatgtccctccaacacag
atccgcaagagtaaaaggaataagagcattcacaaacagagtgaaaaaatcttaagtaccaa
tgggaagcaggaattcctggggtaaaagtatcacttttctggggctggcctaggcatagg
tctctttgctttctgggtggctgtttgaaagtaaaaacctgggttcggcgttaggttc
cgtatctccctggagaagcaccagcaattctcttccctttactgttatagaatagcctga
ggccccctcgtccatccagctaccaccaccaccaccactgcagccagggcctggccttct
cctacttaggggaagaaaaagcaagtgttcactgctccttgctgcattgatccaggagata
attgtttcattcatcctgaccaagactgagccagcttagcaactgctcggagacaaatc
tcagaaccttgtcccagccagtgaggatgacagtgacaccagagggactagaatacgca
gaactaccaggtggcaaagtacttgtcatagactcctttgctaatagcatacaaaaaatt
cttttagagattataacaaatttttcaaatcattccttagatacc

FIGURE 11

Composite FOR16DIII transcript

tj96c08.x1, wb85d11.x1

GCGTAGGGGggccaggtgcctccacagtcagcc**ATG**gcagcgcctgcactacccggaactg
gacgacacggacagtgaggacgagctgctccggggctgggaggagagaaccacaaaggac
ggctgggtttactacgccaatcacaccgaggagaagactcagtgggaacatccaaaaact
ggaaaaagaaaacgagtggcaggagatttgccatacggatgggaacaagaactgatgag
aacggacaagtgtttttgttgaccatataaataaaagaaccacctacttggaccaaga
ctggcggtttactgtggatgataatccgaccaagccaaccacccggcaaagatacgacggc
agcaccactgccatggaaattctccagggccgggatttcactggcaaagtcgttgtggtc
actggagctaattcaggaatagggttcgaaaccgccaagtcttttgccctccatggtgca
catgtgatcttggcctgcaggaacatggcaagggcgagtgaagcagtgctcacgcatttta
gaagaatggaaaacaaaataccaccctccgccagaaaagtgcagaataaaaattttcccc
tagcaaaagaaggaaaaaataaaagatcttgaatagtttcataaaaaaaaaaaaaa

FIGURE 12

Composite *FOR16D* transcript IV

[tj96c08.x1, tm78c03.x1, qi38g12.x1]

GCGTAGGGGggccaggtgcctccacagtcagccATGgcagcgctgcgctacgcggggctg
gacgacacggacagtgaggacgagctgcctccgggctgggaggagagaacaccaaggacg
gctggggtttactacgccaagtaagggggccgcagtggggccgaggacgcacctgggaccc
tgcacagcccacggacgccacctgcgcggggaggacgcgcactccagcgcagcgcgtgcg
gtgcaaagtgaaagtaactgttaaggagcttcagggaaaagggtccagggttcccagtag
gggccgggcccccttggtgggcctcgggtccagcgggggtcacctgggtgccttcccggcgc
gccctctgctgttcaggatgcagcactgcgcggcgcggcgagggcaaagcggcctcatcc
ccgccaaaaaataaagatgttttaaaaagcgcaaaa

FIGURE 13 A

amino acid sequence of *FOR16DI*

[qi38g12.x1, wb85d11.x1 and HHCMA56 and qg88f04]

[MAALRYAGLD] [DTDSEDELLRGWEERTTKDGWVYYANHTEEKTQWEHPKTGKRKR
VAG
DLPGWEQETDENGQVFFVDHINKRTTYLDPRLAFTVDDNPTKPTTRQRYDGSTTAMEIL
OGRDFTGKVVVVVTGANSIGFETAKSFALHGAHV] [ILACRNMARASEAVSRILEEWHK]
/RAKVETMTLDLALLRSVQHAEAFKAKNVPLHVLVCNAATFALPGVSQRWPGDTFQVNH
LGHFYLVQLLQDVLCSAPARVIVVSSESHRFTDINDSLGKLDFSRLSPTKNDYWAMLAY
NRSKLCNILFSNELHRRLSPRGVTSNAVHPGNMYSNIHRSWWVYTLLFTLARPFKSMV
SDCLVEGGHF

FIGURE 13 B

amino acid sequence of *FOR16DII*

[qi38g12.x1, wb85d11.x1 overlap and HHCMA56]

[MAALRYAGLD] [DTDSEDELLRGWEERTTKDGWVYYANHTEEKTQWEHPKTGKRKR
VAG
DLPGWEQETDENGQVFFVDHINKRTTYLDPRLAFTVDDNPTKPTTRQRYDGSTTAMEIL
OGRDFTGKVVVVVTGANSIGFETAKSFALHGAHV] ILACRNMARASEAVSRILEEWHKAK
VEAMTLDLALLRSVQHFAEAFKAKNVPLHVLVCNAATFALPWSLTKDGLLETTFQVNH
LGH
FYLVQLLPGMFCAAQLLPVSLWSPQSPIDLQILTPWENWTSVASLQKKTIGRCWLITG
PSSATSSSPTSCTVASPTRGHVERSDRSWKYDVLQHSSQLVGVHTAVYLGEAFHQVHATG
SCHHRVLCCCPRGTGSRDVLQQLPLHALTRSSERRDGPDPVGLSEPLIQERLAASPAK
WSSERMGTHTRPVCVPSRKCQAGPLPNVPPTQIRKSKGNKSIHNRVKNLKYQWEAGNSWG
KVSFLFWGARHRSCLFLVVACLKVKTWLACRFRISLEKHQQFSSFYCYRIA

FIGURE 13 C

amino acid sequence of *FOR16DIII*

[combination of qi38g12.x1 and wb85d11.x1]

[MAALRYAGLD] DTDSEDELLRGWEERTTKDGWVYYANHTEEKTQWEHPKTGKRKR
VAGD
LPGWEQETDENGQVFFVDHINKRTTYLDPRLAFTVDDNPTKPTTRQRYDGSTTAMEILQ
GRDFTGKVVVVVTGANSIGFETAKSFALHGAHVILACRNMARASEAVSRILEEWHKTKYHP
PPEKCRKIFP HAKAYEA

FIGURE 13 D

complete amino acid sequence of *FOR16DIV*

MAALRYAGLDDTDSEDELLPGWEERTTPRTAGFTTPSKGAAVGPRTLGPCTAH
GRHLRGEDAHSSAARAVQSESNC TTK

FIGURE 14

Amino acid homology sequence motifs

FOR16DIV has a conserved motif possibly connected to DNA replication

sp Q13526 PINI Human Peptidyl-prolyl cis trans isomerase NIMA interacting 1

15	EDELPPGWEERTPRTAG	31
	: : : : : : : :	
4	EEKLPPGWEKRMSRSSG	20

sp P46935 NED4 Mouse NEDD 4

12	TDSED--ELPPGWEERT	26
	: : : : : : : :	
524	TDSNDLGELPPGWEERT	540

sp P46934 NED4 human NEDD-4 Protein (KIAA0093)

9	LDDTDSEDELPPGWEERT	26
	: : : : : : : :	
524	LDTSNLGPLPPGWEERT	540

sp P54353 DOD DROME DODO Protein.

16	DELPPGWEERTPRTAGFT	33
	: : : : : : : :	
5	EQLPDGWEKRTSRSTGMS	22

FIGURE 15

DNA sequence of exons

Exon A

ggccaggtgcctccacagtcagccatggcagcgctgcgctacgcggggctggacgacacg
gacacggacagtgaggacgagctgctccggggctgggaggagagaaccacaaaggacggc
tgggtttactacgccaatcacaccgaggagaagactcagtgggaacatccaaaaactgga
aaaagaaaacgagtggcaggagatttgccatacggatgggaacaagaaactgatgagaac
ggacaagtgtttttgttgaccatataaataaaaagaaccacctacttggacccaagactg
gcgtttactgtggatgataatccgaccaagccaaccacccggcaaagatacgacggcagc
accactgccatggaaattctccagggccgggatttctactggcaaagtgggtgtggtcact
ggagctaattcagga

Exon 1

atagggttcgaaaccgccaagtcttttgccctccatgggtgcacatgtgatcttggcctgc
aggaacatggcaagggcgagtggaagcagtggtcacgcattttagaagaatgg

Exon Z

aaaacaaaataccaccctccgccagaaaagtgcagaataaaaaattttcccctagcaaaag
aaggaaaaataaaagatcttgaatagtttcatcaaaaaaaaaaaaaaaaaaa

Exon W

gtaagggggccgcagtgggggccgcggacgcacctgggaccctgcacagcccacggacgcc
acctgcgcggggaggacgcgcactccagcgcagcgcgtgcggtgcaaagtgaagtaact
gttaaggagcttcagggaaggggtccaggggtcccagtagggggccggccccccttgggtgg
gcctcgggtccagcgggggtcacctgggtggcttcccggcgcgcctctcgtgttcaggat
gcagcactgcgcggcgcggcgaggggcaaagcggcctcatccccgcaaaaaataaagatg
ttttaaaaagcgcaaaa

Exon 2

cataaagccaaggtagaagcaatgaccctggacctcgctctgctccgtagcgtgcagcat
tttgcgaagcattcaaggccaagaatg

Exon 3

gcctcttcatgtgcttgtgtgcaacgcagcaacttttgctctacccggagtctcacaag
atggcctggagacaccttccaagtgaatcatctggggcacttctaccttgccagctcct
ccaggatgttttgtgccgctcagctcctgcccgtgtcattgtgggtctcctcagagtccca
tcg

Exon 4,

atttacagatattaacgactccttgggaaaactggacttcagtcgcctctctccaacaaa
aaacgactattgggcatgctggcttataacaggtccaagctctgcaacatcctcttctc
caacgagctgcaccgtgcctctccccacgcggggtcacgtcgaacgcagtgcatcctgg
aaatatgatgtactccaacattcatcgcagctgggtgggtgtacacactgctgtttacctt
ggcgaggcctttcaccaagtccatg

Exon 5,

gtttcagactgcctggtagaaggagggtcacttctgattgtcagtgactttg

Exon 6

agctgagtgctgaaataaaatgataaacaagtcaaaaa

Exon X

caacagggagctgccaccaccgtgtactgtgctgctgtcccagaactggaggggtctagga
gggatgtacttcaacaactgctgccgctgcatgccctcaccagaagctcagagcgaagag
acggcccggaccctgtgggcctcagcgagaggctgatccaagaacgcttggcagccagtc
cggctaagtggagctcagagcggatgggacacacaccccgcctgtgtgtgtcccctcac
gcaagtgccaggctggggccccttccaaatgtccctccaacacagatccgcaagagtaaag
gaaataagagcattcacaacagagtgaaaaaatcttaagtaccaatgggaagcaggggaatt
cctggggtaaagtatcacttttctggggctgggctaggcataggtctctttgctttctgg
tggtggcctgtttgaaagtaaaaacctggttggcgtgtaggttccgtatctccctggaga
agcaccagcaattctcttcccttttactgttatagaatagcctgaggtcccctcgtccatc
cagctaccaccaccaccactgcagccaggggctggccttctcctacttagggaagaa
aaagcaagtgttactgctccttgctgcattgatccaggagataattgtttcattcatcc
tgaccaagactgagccagcttagcaactgctggggagacaaatctcagaaccttgtecca
gccagtgaggatgacagtgacaccagaggagtagaatacgcagaactaccaggtggca

aagtacttgatcatagactcctttgctaatactatacaaaaaattcttttagagattataac
aaatttttcaaatacttccttagataacc

FIGURE 16

BAC #2 - finished sequence (270kb)

5 GATGGCGCTTTATTATGAGATACACGAAGACAGAGACAGAGGTTCCCAAGTAGCCCTGGACCTGCAGTGTACAGCATC
ACCTGGTAGCTGTGTTACACATAAAAAATTCITGGGCTTTATTCACACATAATAAAATCAAAAGCTTGTGGGGTGGGGCTG
CAGTCTGAGGTTTATTTTTCGAAAAGCTGAAGTTCACAGGTTACATGTGAAAAATGTGCAAGTTTGA AAAATAGGTAATC
ATCTGCCAGCGTGGTATCTGCTCAAAAGATCAACTATCACCCTGGATATGAAGCCAGCTCCAGTACCTGTTGTTACAGA
TGCTCTACCCACCACATGCAAGCCCCAGTGTGTGTTATCCCGCTCCACCGTGTGTCATGTTCTCATCATGCAGCTCC
10 CATTTATAAACGAGAGCATACAGTGTTTGGTTTTCAGTTCCTGCATAAGTTTCGCTTAGGATAACGGCTTCCAGCTCCATC
CATGTCCCTCGAGAGACATGATCTCTTCTCTTTTATGATTATATTAATATCCATGGTATATGTACCATATTTTCTT
CATCTTATCATTTATGAGCATTTGGAGTGATTCGGTGTCTTTCAGTTCATTTGTAATACCAATTTTGACCCAGCAATCCCCATTA
CTGGGTATGTAACCAAGGAATAGAAATGATTGTTATTAAGGTATGTCACATGTATGTTGATTTGAGCAGGTTTTCAC
AGTAGAAAAGACATGGAATTCACCCAATCCGAATTTTAAAGTCTTCCATGTGATTTCTGAGACAGACTCATGTTCCTGT
15 GGTCTCTGGCTCTCGAGAAGCTGAGGTGGGGCCCCAAAAGCTTGCCTTTCTAAAAGGTACTAGCTGAGCATGATGACTCACA
TGTAACTCCACCACCTTTGTGAGGCCGAGGCGGGTGGATGCTTGTAGGCCAGGAGTTTGAGACCCAGCTTGGCAACATGGT
GAAACTCCATCTCTACTAAAAATATAAAAAATTTGCGGGTGTAGTGGTGCACACCTGTAGTCCAGCTTAATTTTGAAGGC
GGGGCAGGAGAATCGCTTGAACCTGGGAGGCAGATGTTGCAGTGCAGTGCAGTGCAGGCACTGCACCTCAACTCCAGGCTGA
CAGAGCCAGAGTCTGTCTCAAAAAACGAAAAACAAAAAAGCTAAATGTATAAAAGGTACCCAGGTGATGTT
20 GATGCTACTTGTCTTGGCCCAATGAGGATGAAAAGCCTACAGGCCAGGCATGGTGGCTCATGCTCTGTATCCACAACCTTTGG
GAGGCTGAGGCAGGAGCATCACTGAAGTCAAGAATTCAAAAGCAGCTTGACCAACATGGTGAACCCCACTCCCTACTAA
AAATACAAAAATAACACCAAAAAAAGAAAAACCAAAATTTAGCCAGGTGTGGTGTATGGCTCTATAGTTCAGCTCCAGCT
ATTTGGGAGGCTGAGGCAGAGAAATCTCTTGAATCCAGGAGGTGAACGTTGCAGTGCAGCAAGATCTCACCAGTGCACCT
25 TAGCCTGAACCTTCAGAGAGAGACTATGTCTCAAAAAAAGAAAAAAGCCCTACAGTTGACAGGCAGATTAACAGAGG
GAGCAAGGAGCTTTGTGGAGACAATAGGGAGTGTTGAGGACCTGTCAAAACACACAGTCCCCATCTGTACTTTGGGAG
GGAAGCACTTCTTAATTCAGCTGCCTGTGTGCCATGGGGGAACATGACAGCCAGACTGCCATCTCTTCTTAATTTTPTAA
AAATTTAAAGGCTAAAAGTTTGGCTTATAAAAAATGGTATTTCTTGAGTTTTTTACAATTTATAGTATTAAGATATTCCTAG
GTTTTTGTGTTGTTTTCGAGACGAAGTTTTCGCTCTCATTTCCAGGATGGAGTGCAGTGTGTGAATCTCTCTCGCCCTCCCA
30 GTTTAAGCCATTTCTCTCGCTCAGCCTCTGAGTAGCTGGGATACAGGCGCCCGCCACCATGCCCGGCTAATTTTPTGT
ATTTTATGATACAGCGGGTTTTCAGATGTTTGGCCAGGCTGGTCTCAAACTCTGACCTTCAGATTCACCCAGCTCCAGCTG
GCCTCTTAAAGGTTGGGATTACAGGTGTCAGCTAGTGCACCTGACCTTAGGATAATCTTAATTTTAAAGTAAGTGGTAAC
TCATAAAATTTTAAACACTCTGCAGTCAAAAGAAAACATGTTTGTGGTAGGTAGCCAGTTTATTAACCTGTGTGAAGACTGT
35 GTTATTAGCAAAATCCCTCAATGTGTGACCAAGAGTGGGTAGGAATCTTCGACAGACAATCTTTTGAAGGTGGTTTGT
TTTTATTCTCTTATAGTACAGTTGTAGATTATGAAATAGTATGATATAGTAAAGGTTCTGTGTACCCAGCAT
CGCAATTGAGCATATAGTACATGCTGTCTGTGTACCCAGTATCCCTGGTCTTGCCTCTGGTATGAGGGTATTAACATC
TTGCAATGATGTGTGCACATTTGCTTACAATTAATAAACCAATATCGATACATTTTAACTAGTCTATAGTTTACATGAGG
40 GCTGTCTGTGTGTGTATCTTATAGGTTTGTTCATAGGTTATGATGACAGGTGTGTCTGTCTTATGAGGTGAAAC
CATTCCAATTTTATACAGCGGTATTTTACGCCCTTAAAGTTCCTCGAAAGTGTTATTAAGAGTACTACTTGCAGCTGTC
CTCATTTCCCCAGCAAAGTCTTCTTGAGAAATTTAGGCGCTTTAGGCTAGAGGATCTCAGTGTCTGACAAAAGCGGTATG
45 ATTGTGATTTTGATTTTGGATTTTCTTGATGGCTTAGGATATGAACATCTCTTCATATGATTATTGGATGGATGATTTT
TTACGCGGTTATCAGGTCACACCTGTAATCATGCCCCACCTTCATTTTCTTACAGTGCAGTGTGTGCTTTTAGGCACATACC
AAGAGCTTACAGAGACTCTTCTGTTTACAGGAAAGATTCACATCCCCAACAAACACTGCTCCCCGATTCACATATAGAG
AGGCTAAATTTATGTCCATATCACTTTTCTTPTAAATCCCTTTACTTTTGTGTCTACAAGAGTTTTCAGAAATTAATATC
50 TGCAGAAAAAAGTACTGATTTGCTCAGAGAGTGTGTGACAGGTAGAAAACGGGATGCCGACTGCATTGGTTTAGCCAGAAC
TGTGGCCATGAAACCTTGCTTGGCCCGCAGGATGGGGTGGGGGGATATCATATAGCTTTTAGGTGGGGCAGAGGGGT
GGTCTGCCCTTGTGCAGCGAGGAATGTTACGAAGCAGGCTGCCTCTGTTTCTACTTAAATTTTAAATTTACAGTTACCGATG
GCATGGATCTTGACTAAGCGGAGCAGATGTGGCTCTGTATTGACATAGCTCTGAAATATCAGCCCTGCTTGGCTATG
ATGTGTTTGTGTGTGTTTGTAGACAGAGTCTCGGTCTCTTGGCCAGGCTGGAATGCAGTGGCAGATGTTGGCTCACT
55 GCAAAACCTGCTCCACAGGTTCAAGCGATTTCTCTTCCACAGCCTCCCGAGTCTTGGGATTCAGAGCTGCATACCAG
GCCAGCTTAATTTTATATTTTACTCGAGAGCTAGTTTCCGCATGTTGGCCAGGCTGGTCTCAAACTCGTACCTTTAG
TGATATGCGCCCCCTCAGCTTCTTAAAGTGTCTGGGATTAGAGGATAGAGCCACAGACATGACCTGTGTTTATGTTTAA
ATCACCATTGTCAATCTTGAAAGGAGACTTTGTTTGTGATGTATGAAGATTGAGTTTCTTACTATCTCTACATAGTTTCC
60 TCAGATTGCTTTTPTTTTGTGAGACCGAGTCTAGCTCTGTCAACCCAGCTGGAGTGCAGTGGCACAATTTTGTCTCA
CTGCAACCTCTGCTCCCATGTTTTCAGTGATCTCTGCTTCAGATTCCCGTGTACAGGCTGCCACCACCTTGGCTAA
TTTTTGTATTTTGTAGTAGATGGCATTTTGGCATGTTGGCCAGGCTGTCTTGAACCTCCCGACTCAGGTGATCCACTC
GTCTAAGACTCCCAAAGTGTCTCGGATTACAGATGTGAGCCACTGCACCCAGCATTTCTTTAGATTTCCAATAAAAATAAAA
AGCTGTGTGGGAAGTCAGAACTTTGGTTCATGTCATATTTCTTATTTTAAAGATTACAGATATTAACGACTCTCTTG
65 GCAAAACCTGGACTTCAGTTCGCTCTTCCAACAAAAACGACTATTTGGCGGATGTGGCTTATAACAGGTTCCAAGCTCTG
CAACATCTCTTCTCCAACGAGCTGCACCGTGCCTCTTCCAGCGGGGTACGTCGAACGAGTGCATCTCTGGAATA
TGATGTACTCCAACATTCATCGCAGCTGGTGGGTGTACACACTGCTGTTTACCCTTGGCGAGGCTTTTCAACAGTCCATG
GTAAGAGAACAGCTTCTGGCGCGGCAAAACACTTGGGTCTTAGAGAAACCTGCACATGTGTCTCCACCTTTTACCCTC
TTGCGGGCATGAGTCTGGTCTCAGTAATAACATATGTTCCAGCCCATCAAAAAGGGCTCTTGAACATTTTTCATCAACTT
AGGTTAAGTCTGTTTGGGTAAGTGCCTCTGGAGGCTGGGTAGAAGATGGGGTTTTCAGTATCATGTTAAGTATGGCT
AAAGTCTTTATGGAATGGTGAATTTTTTGTGTTGTTTGGTTTGTGTTTTTGGGGTTTATTCAGAAATTTGAAAA
70 TCTATTTTGTGGAATGAGCACTTGAAACATGCTGTTTGTGTGCTAGTGGTAAACAACAAACATTTGGTGACTACTGAATT
TTTCAGCAGATGTGATTTGTTTGTCTCAGAAAAAAGTGGATCTTTTGTGTTTCTAAATTTTCTTCTTAATGGGTATAATCC
TGTGTGAGAGTCTTTGATAGCTACGAGTGTGTTTCTTCTTCTTCCAAAGACAATTTTGGATGAATCATGGTA
CTGTGGTTACATTTGGAAGTGTTTACAAGGTGATAAGATGTTTATAGTTTGGTGTCTATTATTCAGCAATATTAAGA

CGGGTAAATCTGCCCTTGAATAACCCGCTGCAGGGTGATTTATAGTTGTACAAATTTAAACGTAATGAAGGAGATAATA
GATGTTGATTTGTTCCTGAAATTTAGTATTTTGTAGAAAATACCTTCTCCCCCTTTCCATCTTTAAATACCTGACGCTGACTA
CACTATAGATACATATCAAGGGTAAAGCCCCCTGTTTGAATGTGTATTTGGTACATTTGATATACATACAGGCAAGGCG
TGTATAAAACAGGCTTTCTGTTGCGAGGGGAAATATGGTTTTCAAAGTGGAATTTCAATGTATCTTCATGAATCTAA
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[illegible]

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[illegible]

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FIGURE 18

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